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THE JOURNAL OF HYGIENE

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THE JOURNAL OF HYGIENE

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NOTES ON SOME TROPICAL DISEASES IN
PALESTINE.*(Continued from Vol. XIII. No. 1.)*

By E. W. G. MASTERMAN, M.D., F.R.C.S., D.P.H.

(With Plate I and 1 Chart.)

III. *Enteric and Typhus Fevers.*

UNTIL recently there has been a general impression among a large proportion of the local medical faculty in Jerusalem that enteric fever was very uncommon and that typhus fever was practically unknown. As we are now basing our diagnosis of malaria increasingly upon the discovery of malarial parasites in the blood, we find that while on the one hand many of the most severe malarial infections, especially those with subtertian parasites, have little or no pyrexia, on the other hand, many cases which we should have called "remittent malaria" or "malignant malaria" or "typho-malaria" in years gone by, are proving to be of other origin. A case of remittent pyrexia which does not yield in three, or at the outside five, days to efficient dosing with quinine is not in our experience a pure malarial infection.

Enteric Fever.

With regard to enteric fever there has not been a single year, during the 20 odd years in which I have been connected with our hospital, in which we have not had well-marked cases in our wards. In looking through our records of the decade from 1903-12 I find we had 60 cases definitely diagnosed as enteric. Of these 60 cases—36 males and 24 females—three died, one of acute dilatation of the heart (she had mitral disease), one of haemorrhage, and one (probably) of perforation.

Of the total 70% were 20 years of age or under. It is possible that one or two typhus cases may be included, but it is certain also that some of the more prolonged "remittent" fevers in children which we considered in the earlier years to be malarial were mild enteric. Cases are by no means rare with an "intermittent" temperature throughout. Now that we are having systematic examinations of the blood and excreta at the "International Health Bureau" we find such atypical cases occur. It seems probable that the comparative rarity of enteric fever among the middle-aged native-born population is largely due to its not uncommon occurrence in childhood. It should be pointed out that the statistics of in-patients are no real criterion of the actual prevalence of enteric fever because there is, as a rule, such a strong prejudice on the part of many members of the native population—Jewish and Moslem—against sending their friends to a hospital when acutely and dangerously ill that a large proportion of these cases are treated at home where under the conditions of medical practice here—which often means a new doctor every two or three days—they never get properly diagnosed. Here again Widal's test or bacteriological examination of the excreta is proving of great assistance for speedy and accurate diagnosis. During the present year we have had three cases of enteric in the hospital, in all the diagnoses were confirmed by the Widal test.

Among the British residents in Palestine, enteric fever is certainly not as frequent as in Beirût and Cairo—with their public water supply and town sewers—but the eight cases I have personally known of, have all been severe attacks and three were fatal. With reasonable care it does not seem to me that European residents and visitors, living under favourable surroundings, run very much risk of acquiring enteric fever in Jerusalem; in this respect this city compares most favourably with Cairo.

I am informed by the assistant-physician of the large German hospital here that they have from 15 to 20 cases of enteric fever in their hospital annually but the majority of these are not from Jerusalem itself, many are from Jaffa and others from villages between Jerusalem and Jaffa. In 1908 when there were 30 cases, no less than 14 were from one family in Ramallah. The majority are natives—Moslems or Christians—but a certain number come annually from among the poorer German colonists and from what we should call tramps. All those cases which have been in this hospital in the last year have yielded a positive Widal reaction.

Typhus Fever.

Typhus fever is probably a very much commoner disease in Jerusalem than has been supposed. Cases usually occur during the colder months. During the last cold season we had seven such cases in our hospital and I saw others in the homes. Dr Canaan, an able Syrian doctor with a large private practice, says that he attended 25 undoubted cases—chiefly Moslems—in the winter (1912–13) and other doctors have had a similar experience. In the cases briefly detailed below it will be noticed that three of them were members of our staff infected from the patients; a few years ago one of our English nurses also caught the disease but happily recovered.

As there is still a certain amount of scepticism regarding the prevalence of typhus—these cases being sometimes called “malignant, remittent malaria,” etc.—I give below in brief outline the main symptoms of our seven in-patients.

Case I. S., a Russian Jewish immigrant, aged 19, admitted Nov. 30, 1912, in a semi-dazed condition with a temperature 102° F. He was unable to give any clear account of himself but we gathered that he had probably been ill for one week. During the next week his temperature was mostly below 102° , but once reached 104° . His mental condition was confused. Bowels constipated (had to be treated by enemata, to which he offered violent resistance). Slight rash on abdomen not unlike “rose spots” but far more plentiful. Urine: no albumen. Temperature fell to normal on what we calculated must be about the 13th day—seven days after admission—and, after a rise to 99.4° next afternoon, remained down. Good and rapid recovery.

Case II. B., a Spanish Jewess, aged 45, employed as a ward servant, was ill with “fever” two or three days before she took to bed. Seen in bed Dec. 4, 1912, with severe headache, aching all over, etc. and supposed to have, the then prevailing disease, dengue. Temperature, first day in bed, 101° ; rose gradually, 104° (8th inst.); 104.2° (9th); 104.6° (10th); and 105° (11th). In searching for “rose spots” a general mottling of the skin of abdomen and chest was seen but not at the time noted as the case was supposed to be enteric. Dec. 10th, muttering delirium, stools very offensive; 11th, patient very drowsy, headache better. Temperature fell by crisis on Dec. 15th—the 13th day—from 102.2° to 99° ; next day rose once to 100.2° and then remained down. Blood examination negative: Widal’s reaction negative.

Case III. Z., Spanish Jew, aged 10, admitted Dec. 11th, 1912, with fever of some days duration. Temperature 103° on admission, 104° next day. Pulse 120–130. Abdomen retracted, no “rose spots,” no rash noticed, lungs clear. Urine: no albumen. Blood negative to malaria. Widal’s reaction *positive* (this rather upset our diagnosis but must have been a result of a *previous* attack of enteric). On the sixth day after admission, temperature fell by crisis from 101.4° to normal

and except for one rise at 7 a.m. next morning to 100°, it remained down. Rapid and complete recovery.

Case IV. M., Spanish Jew, aged 4, brother of Case III, admitted Jan. 6, 1913, with fever of some days duration. Temperature 103·4° first day; rose to 105° second day, but afterwards remained between 101° and 103° till the eighth day after admission, when it fell by crisis to 98° and, except for one rise to 99° next evening, remained down. A slight evanescent rash observed. Constipation throughout. Urine normal. Blood negative to malaria and enteric tests.

Case V. D., a Spanish Jewess, aged 28, mother of Cases III and IV, admitted Jan. 8, 1913, with history of six days illness. Aching all over, tongue dry and furred, very constipated, no cough, no rose spots, a slight vesicular eruption noted about waist. Urine: considerable cloud of albumen. Very drowsy and indifferent to her surroundings—this increased after crisis. Subtulus tendinum marked. Pulse 112 to 140, very weak. Temperature fell by crisis, on ninth day after admission, to 99°; next morning rose to 100°, fell by noon to normal and remained down. Albumen disappeared from urine at once; pulse, however, became alarmingly weak and irregular after crisis, but patient left quite well three weeks later.

Case VI. R., a Spanish Jewess, aged 45, employed as servant in the hospital, taken ill with "fever," Feb. 3rd, 1913, temp. 101° to 102°; next day fell to 100°; following day reached 104° but later almost always below 102° till the end. Profuse typhus rash, at first an erythematous mottling of the skin, well marked, later widely diffused petechiae; horrible faector; urine loaded with albumen. Blood negative for malaria and enteric. On the seventh day of the fever patient got rapidly worse, respirations ran up to 56. Eighth day, violently delirious, pulse 140, respirations 60; ninth day, pulse 144, respirations 64; patient semicomatose; died at 8 p.m.

Case VII. Miss W., an English nurse, aged 37, had "fever" for two days before being put to bed (Feb. 5th) with temperature 102°; rose by seventh to 104° and remained generally between 102° and 104° next ten days. Rash and faector characteristic and marked. Heart very irregular—no valvular disease. Pulse never above 120, after crisis rapidly fell to 70–80. Condition last two days before crisis seemed very critical. On Feb. 17th (14th day of illness) temperature fell from 101° to 97·4° and after rising to 99° at six next morning remained down. Unbroken and rapid recovery. Blood examination (at beginning of illness) for malaria and enteric, negative.

I think these brief notes are sufficient to convince anyone that we were here dealing with a small epidemic of typhus. All the cases that recovered had a typical crisis about the 14th day; in all but one Widal's reaction was negative; the rash was distinct in three cases and would no doubt have been noticed in the earlier cases—although it must have been very faint—had we not diagnosed these (supported in one case by a positive Widal's test) as enteric; in the severe cases the faector was strong and overpowering. As usual, age had a great bearing on the severity of the symptoms—the one fatal

case was the oldest. In looking through our hospital records I find that there were during the last 10 years—previous to this epidemic—eight cases among the in-patients diagnosed as typhus, of which three were fatal. In the more squalid parts of Jerusalem, within the old city walls, epidemics of typhus are not uncommon, but such cases, if only seen under the difficult conditions of their home surroundings—in semi-darkness, amid filth and rags—are liable to be confused with malaria, enteric, pneumonia, etc.

IV. *Jericho boil.* (Plate I, fig. 1.)

It is a fact, long familiar to the inhabitants of Southern Palestine, that every new-comer visiting Jericho during the summer months—and especially during August and September—is liable to acquire a troublesome skin affection which lasts as a rule for six months and may last for a year, or more. The residents in the little village of Jericho having all had this disease—in childhood if born there—are all now immune, but it is said that practically every new-comer in the summer gets the disease. I myself had occasion to visit Jericho in the hot season and soon got half a dozen “boils” scattered about my legs and arms—fortunately not on my face—which lasted several months and have left small scars.

The scars left by the Aleppo “button” or “boil,” the Syrian variety of Oriental sore, are very familiar to us as we have many hundreds of Aleppo Jews in Jerusalem, and I never saw anyone who has resided long in that city who could not point to the scars. Indeed, in the case of those infected as young children, the scars, often very extensive and unsightly, are usually upon the face. The same is the case with the “Baghdad boil.” Occasionally we have people coming from one of these cities with their Oriental sores unhealed. One patient of mine went to Aleppo to keep the Passover feast with her relatives; she was only there a few days but acquired a sore which it took months to heal. My friend, Dr Adams, of the Syrian Protestant College, Beirût, has had great success in treating these Oriental sores with solid carbon dioxide. Now, the “Jericho boil” presents some clinical features distinct from the Oriental sore as we see it acquired in Aleppo and Baghdad. I have long been inclined to think that it is a disease *sui generis*. It has the features, common to such sores, firstly, of belonging geographically to one locality. It seems to be peculiar to the village of Jericho itself, and to a few neighbouring places in the Jordan valley;

secondly, it seems to attack all those not immunised by previous attacks; and, thirdly—until recent methods of treatment—its duration has always been long, several months at least. On the other hand, it differs from the ordinary Syrian Oriental sore as follows:

(1) In my experience it is the rule that the lesions are far more numerous. The Aleppo boil is solitary or at most there are three or four: in the case of the "Jericho boil" the lesions may be as many as a dozen and are frequently very many more.

(2) The lesions of the Jericho boil are *as a rule* more superficial in character and do not lead to such deep scarring as the Syrian Oriental sore. I have seen one or two exceptions to this: *e.g.* one small girl at Jericho who had a scar on her nose very like that of an Aleppo boil, although she had other more superficial scars besides, and an old Moslem woman, in debilitated health, who had a very large and septic sore on the back of the hand, which I think was due to a secondary infection with septic microorganisms.

(3) My friend, Dr Huntermüller, of the "International Health Bureau," has, at my suggestion, made a very exhaustive examination of some skin sections which I excised for him from Case II and he finds no Leishman-Donovan bodies but certain other bodies of a distinctive character. A report on these pathological appearances will be published later when more material has been examined.

Jericho is a small place, with 300 inhabitants, and not greatly visited by the susceptible during the summer months, so that cases of this disease are not common. In a special visit which Dr Huntermüller and I made this summer for the purpose of getting material, we only saw four cases and of these two were practically healed. A couple of years ago I had under my care the family of a Turkish official whose duties had necessitated his residence in Jericho for many months. All the members of his family returned to Jerusalem infected with these "boils" but all, except in the case of the old lady mentioned above, healed after a few months of treatment.

The two following cases were in-patients in my hospital:

Case I. A., a Jew from Yemin, aged 20, was in Jericho in July, 1905, for one month and came to the hospital two months later with the following lesions. He had thirteen "boils" on his face; eleven on the front of his chest; twelve on the upper part of his back; eight on his right lower leg—three of which were large and crater-like—these were the first to appear; seven on his left leg. My description, written in the clinical notes at the time, is: "The spots began as small raised papules, pink in colour and varying in size from a pin's head to a considerable size:

there is a certain amount of desquamation over the papules and when scratched, as for example on the hands, they have become small, suppurating, ulcers. The larger spots are about the size of a sixpence, they are flattened out and ulcerated at the centre, nothing can be squeezed out of them. The lesions are quite superficial and there is very little induration around. They have little or no scab on the surface." The patient was treated for a few days with arsenic internally and an ointment of zinc and mercury, but he would not stay in the hospital and has been lost sight of.

Case II. S., an Aleppo Jewess, aged 17, admitted to the hospital, Feb. 3, 1913, with a large crop of "boils" consequent upon residing a few weeks in Jericho the previous autumn. The lesions were similar in character to those described above, and I send a photograph which may give some general idea of their appearance (see Pl. I, fig. 1).

The lesions were situated as follows: one at external angle of left eye; one on the left side of the nose. Seven on the right arm, one of which was nearly one inch in diameter, one above right elbow; two at left elbow, two at left wrist and one at the back of the left hand, five near the right knee and one in front of ankle; two above left ankle, one in front of left foot and one at back of left heel.

This patient was, through the kindness of the Director of the International Health Bureau, who supplied the drug, treated by intramuscular injections of neo-salvarsan: 0.1 of a gramme being given at intervals of a few days. Four injections were given with marked improvement. Healing commenced after the first injection and was complete in a few weeks.

It seems probable, from the situation of the lesions, that many of them are produced through auto-inoculation by scratching.

Since writing the above I have had four more cases under my care all presenting the same general symptoms. Two, who were brothers, were Moslem fellahin from Siloam, and had spent some weeks shortly before among the bedoin on the banks of the Jordan some miles north of Jericho. They told me that all the bedoin living in that region had at some time suffered from that disease. One of the brothers altogether refused to submit to treatment with neo-salvarsan, saying that he knew the sores would in any case get well in a few months and that all he wanted was a little ointment. The other brother was admitted to my Hospital, had an intramuscular injection of neo-salvarsan (0.3 gramme) which he found very painful, and when some days later we proposed to give him a second injection intravenously, he took flight and left us rather than submit to further treatment.

The other two cases were Jews, a father and son, who had spent several weeks in the summer at Jericho and came to me with very many lesions. The son refused to come into the Hospital but the father was admitted. I excised one of the lesions under cocaine for microscopic examination and gave him an intramuscular injection (0.3

gramme) of neo-salvarsan. He too felt much pain from the injection and either on account of this or, as he said, on account of his business, he left for Jericho two days later. I was therefore unable to give systematic treatment in any of these cases.

I understand that many Russian pilgrims acquire this disease on their visits to Jericho and the Jordan, and we hope that during 1914 the treatment of these cases with salvarsan or neo-salvarsan will be given a thorough trial in the Russian Hospital.

V. *Ulcerative stomatitis.* (Plate I, fig. 2.)

There is a form of ulceration of the mucous membrane of the mouth which I have observed for several years and which has proved very resistant to ordinary remedies. Cases we have had improve considerably while under hospital treatment but relapse on going out. It occurs commonly in children and young people, sometimes also in pregnant women. The lesions consist of elongated ragged ulcers with deep red edges and sloughy whitish bases, which form especially at the junction of the mucous membrane of the gums and that of the lip; also, commonly, as an irregular line on the inside of the cheek opposite the line of junction of the molars. When the ulceration is deep the affected lip or cheek is often markedly swollen. In more severe cases the alveoli of the teeth are involved and portions become necrosed; indeed some of these cases are actually an early stage of noma. The breath in all these cases is foul; if there is necrosis it is naturally very foul indeed.

In connection with these cases two important points were determined with the assistance of Prof. Mühlens of the "International Health Bureau":

(1) A case of this nature which was in our hospital nearly a year ago had gone on until it assumed the characteristic appearance of *Cancrum oris*. The child, aged 15 months, was a Jewess from Urfa in Northern Syria and on admission was found to have a large and deep ulcer connected with the upper lip. Soon after admission a piece of necrosed upper jaw with three incisor teeth attached was removed but the gangrenous process spread rapidly and the whole upper lip and left cheek were progressively destroyed, the child dying 17 days after admission. On consulting Prof. Mühlens about the case, soon after its admission, he suggested taking a smear from the edge of the ulcer: this when stained with Giemsa's stain showed what looked like a pure



Fig. 1. Case of "Jericho Boil."



Fig. 2. Case of Cancrum oris due to *Spirochaeta vincenti*.



culture of *Spirochaeta vincenti* and *Corynebacterium fusiforme*. Subsequently I took a number of smears from cases such as are described above—which appeared to me to be the earliest stages of noma—and in all those with these characteristic symptoms we found the same microorganisms. Other ulcerations of the mouth show on the smear merely enormous quantities of mixed bacilli and occasionally a few teeth-spirochetes. Thus it would appear as if this particular form of ulcerative stomatitis is due to these special organisms. Whether one is the primary agent and the other of no directly pathogenic importance or whether they are only pathogenic when acting together is not yet clear. Since we made these observations I have learned that similar ones have been made elsewhere¹.

(2) At the suggestion of Prof. Mühlens—who kindly supplied the drug—we commenced treating these cases with neo-salvarsan. The unfortunate child with noma had four intramuscular injections (0.1 gramme) of neo-salvarsan: although the spirochetes disappeared almost at once from the wound, the utmost that the drug accomplished was, probably, some prolongation of the patient's life—a doubtful benefit under the circumstances. But in a whole series of cases which we have had since this treatment has proved most successful; the spirochetes disappear or greatly diminish after the first injection and healing is only a matter of a few days. One case came back with a recurrence after some weeks and the second course of injections did not seem to have so rapid a result either in causing disappearance of the microorganisms or in producing healing. I am told that in some cases of this nature the application of a solution of salvarsan to the surface of the ulcer itself has proved successful. The case in question healed without great delay so I have not yet had occasion to try this mode of treatment.

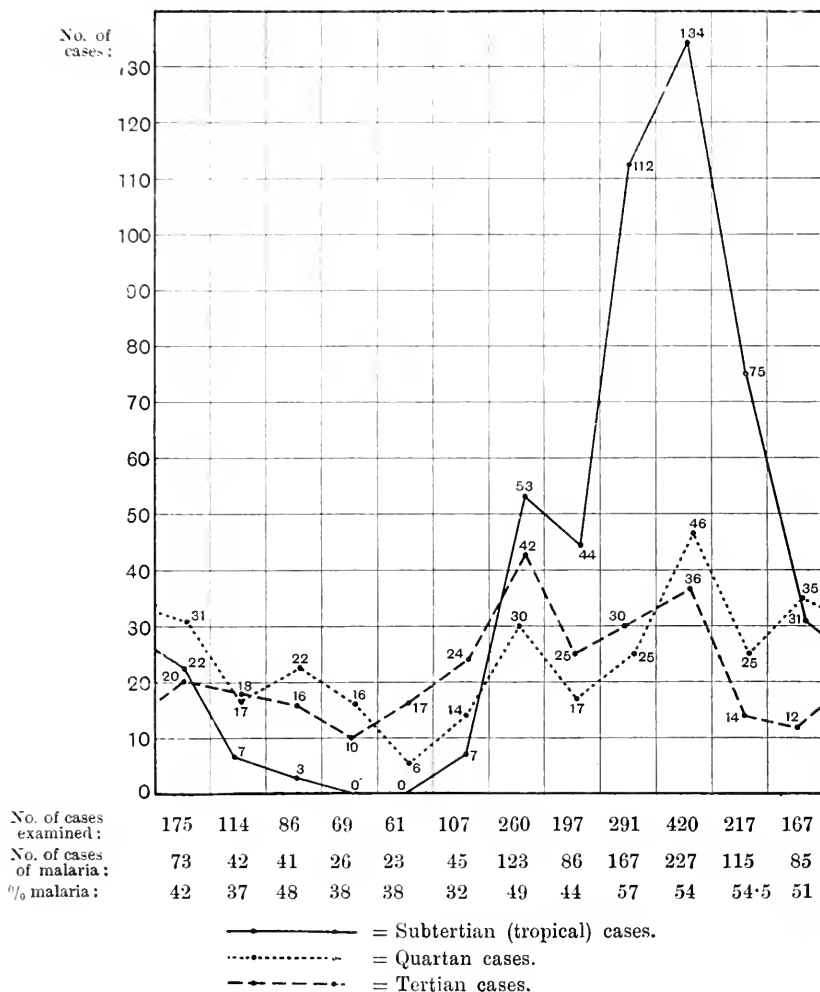
SUPPLEMENTARY NOTE ON MALARIA.

In the *Journal of Hygiene*, Vol. XIII, No. 1, I gave some account of the "Malarial fever of Palestine." I now send a chart to illustrate the results of a systematic blood examination of our "fever" cases, made for a whole year commencing Sept. 24, 1912. Dr Corbitt, my assistant, and I, have as far as possible taken the blood of all new cases coming to our out-patient clinics from week to week. The slides have been stained for me at our "International Health Bureau" and have been

¹ See *Atlas und Grundriss der Bakteriologie* (Lehmann Medizin. Handatlanten), vol. x. pp. 577–579, 679.

The results of blood examinations of "fever" cases attending the clinics connected with the English Hospital, Jerusalem, during a whole year commencing Sept. 24th, 1912.

JAN. FEB. MAR. APR. MAY JUNE JULY AUG. SEPT. OCT. NOV. DEC.
 Mean atmospheric temperature, Fahr., per month:
 49° 46° 54° 61° 65° 74° 76° 76° 75° 68° 60° 52°



systematically examined by Professor Mühleus or, after the first few months, by myself. Unfortunately through our having had a Medical Congress in Jerusalem in the middle of August, neither I nor my assistant was able to take blood preparations for about ten days at that

time. In consequence of this our total numbers that month are at least one-third less than they should be and the rise in the number of cases of all forms of fever, which certainly occurred in other years, appears as a fall. To make the chart more in accordance with the facts about one-third ought to be added to the numbers for each kind of fever.

It is noticeable how far more prevalent is malaria during the latter half of the year. Thus, out of a total of 2166 cases examined during the year, there occurred 612 cases with 250 "positives" (41 %) between 1st of January and 30th of June whilst there were 1554 cases—with 812 "positives" (52 %)—during the latter half of the year. These figures do not in any real degree represent the actual number of malaria cases under our care as during the whole hot season "positive" cases were attending the clinics regularly for treatment, but they were, as far as possible, only registered as such on their first attendance. Thus for example in November, 1912, the number of cases examined were considerably less than in October, although the actual number under treatment for malaria was still high (see *Journal of Hygiene*, Vol. XIII, p. 55, Chart).

The most striking feature of the Chart is the entire disappearance of Subtertian (tropical) malaria during the early Spring. The few cases occurring in February, March and June are usually importations from the hot maritime plains or from the Jordan Valley, where this disease occurs in all seasons. It is noticeable however that all malarial cases diminish in the cool rainy season, when mosquitos are very scarce.

Correction. Dr Cropper informs me that the list of Anophelenes of Palestine I quoted in my earlier paper (Vol. XIII, p. 53) is incorrect in that (a) I omitted *Pyretophorus superpictus* and (b) *P. palestinensis* = *Cellia pharoensis*.

ON THE PROTECTIVE AND CURATIVE PROPERTIES OF CERTAIN FOODSTUFFS AGAINST POLYNEURITIS INDUCED IN BIRDS BY A DIET OF POLISHED RICE.

PART II.

By E. A. COOPER, *Beit Memorial Research Fellow,
Lister Institute of Preventive Medicine.*

IN a previous communication (Cooper, 1913) the amounts of various raw foodstuffs, *e.g.* beef, heart-muscle, brain, fish, egg-yolk, lentils, and barley were set forth which were sufficient to prevent polyneuritis for a definite period in pigeons fed on polished rice.

It was found that, while (ox) cardiac muscle, egg-yolk, lentils, and barley were very efficient in preventing polyneuritis, the voluntary muscle of both ox and fish possessed only feeble anti-neuritic powers. Sheep-brain was less efficient than ox-heart, but more so than beef in preventing polyneuritis.

The relatively small efficiency of brain material suggests that either the anti-neuritic substances, although essential for the integrity of the nervous system, are not present therein in the active condition or they are combined in such a form that they are not readily absorbed from the alimentary canal.

It was also found that, although small amounts of brain material did not prevent polyneuritis, they were effective in checking the loss in weight that usually accompanies the disease. This fact supported the conclusion reached by Schaumann (1911) that the loss in weight was not merely due to the deficiency in polished rice of the anti-neuritic substances, but resulted largely from a secondary deficiency of other substances essential for the maintenance of body-weight.

To obtain more information as to the relative efficacy of various foodstuffs for preventing beri-beri and to ascertain further facts as to

the distribution of the anti-neuritic substances in animal tissues, I have made some more experiments with brain, liver, milk, cheese, nuts, and malt-extract and the results obtained are set forth in the present communication.

These experiments consisted in determining the daily amounts of the above foodstuffs which prevented polyneuritis in pigeons for a definite period. As pigeons develop symptoms of polyneuritis in about three weeks when fed exclusively on polished rice, the standard time selected was 50 days.

Pigeons received daily by artificial feeding constant rations of polished rice equal to 1/20th their initial body-weight and rations different for each bird of the tissue under investigation. All the tissues were thoroughly minced before being fed to the pigeons.

In this way it was possible to determine a maximum daily amount insufficient to prevent polyneuritis for 50 days and a minimum amount sufficient for this purpose. The suitability of the various diets for the maintenance of body-weight was also studied, the birds being weighed weekly for this purpose.

I. *Control experiments with polished rice.*

Seven pigeons were fed artificially on polished rice, the daily ration being 1/20th their initial body-weight. The results of the experiments are set forth below.

TABLE I.

	Pigeon 326	Pigeon 327	Pigeon 328	Pigeon 329
Effect of diet on pigeons	Symptoms of polyneuritis appeared on 23rd day	Symptoms of polyneuritis appeared on 25th day	Symptoms of polyneuritis appeared on 20th day	Symptoms of polyneuritis appeared on 23rd day
Change in weight by end of exp.	- 26 %	- 24 %	- 17 %	- 22 %
	Pigeon 330	Pigeon 133	Pigeon 134	
Effect of diet on pigeons	Symptoms of polyneuritis appeared on 22nd day	Symptoms of polyneuritis appeared on 22nd day	Symptoms of polyneuritis appeared on 17th day	
Change in weight by end of exp.	- 16 %	- 22 %	+ 3 %	

The results indicate that the birds developed symptoms of polyneuritis in about three weeks and at the end of that time had usually lost considerably in weight.

*Polyn neuritis in Birds*II. *Ox-brain.*

TABLE II.

Series I. (a) Cerebellum (water-content 80 %).					
	Pigeon 10	Pigeon 11	Pigeon 12	Pigeon 13	
Daily ration of natural tissue	4 gms.	5 gms.	7 gms.	9 gms.	
Effect of diet on pigeon	Symptoms of polyneuritis on 21st day	Still healthy on 31st day	Still healthy on 31st day	Still healthy on 31st day	
Change in weight by end of exp.	0	- 9 %	0	- 12 %	
(b) Cerebrum (water-content 80 %).					
	Pigeon 6	Pigeon 7	Pigeon 8	Pigeon 9	
Daily ration of natural tissue	4 gms.	5 gms.	7 gms.	9 gms.	
Effect of diet on pigeon	Symptoms of polyneuritis on 22nd day	Still healthy on 31st day	Still healthy on 31st day	Still healthy on 31st day	
Change in weight by end of exp.	- 20 %	- 19 %	- 9 %	- 4 %	
Series II. (a) Cerebellum.					
	Pigeon 340	Pigeon 341	Pigeon 342	Pigeon 343	Pigeon 344
Daily ration of natural tissue	3 gms.	4 gms.	6 gms.	9 gms.	12 gms.
Effect of diet on pigeon	Symptoms of polyneuritis on 24th day	Symptoms of polyneuritis on 34th day	Weakness in limbs on 46th day	Died on 39th day with symptoms of weakness	Still healthy after 50 days
Change in weight by end of exp.	- 15 %	- 17 %	- 6 %	- 8 %	- 5 %
(b) Cerebrum.					
	Pigeon 345	Pigeon 346	Pigeon 347	Pigeon 348	Pigeon 349
Daily ration of natural tissue	3 gms.	4 gms.	6 gms.	9 gms.	12 gms.
Effect of diet on pigeons	Symptoms of polyneuritis on 30th day	Weakness in limbs on 46th day	Still healthy on 50th day	Still healthy on 50th day	Still healthy on 50th day
Change in weight by end of exp.	- 25 %	- 20 %	- 18 %	+ 5 %	- 17 %

The results indicate that the amounts of cerebrum and cerebellum required to prevent the *acute* symptoms of polyn neuritis in pigeons fed on polished rice were about equal, but, while 6 gms. of cerebrum were sufficient to keep the birds healthy and strong for 50 days, 12 gms. of cerebellum were necessary to prevent signs of weakness in the later days of the experiment. Cerebrum therefore appeared to contain

a somewhat greater available amount of the anti-neuritic substances than cerebellum. Notwithstanding this however there was a tendency for cerebellum to be somewhat more effective than cerebrum in reducing the loss in weight resulting from the polished rice diet. These observations support the conclusion reached by Schaumann (1911) that the loss in weight accompanying polyneuritis is not entirely an effect of the malnutrition necessarily resulting from the deficiency in the diet of the anti-neuritic substances, but must be largely due to a secondary deficiency in polished rice of substances essential for the maintenance of body-weight. This view was also supported by the fact recorded in the previous communication (1913) that the addition of a small amount of sheep-brain to the polished rice diet, although ineffective in preventing polyneuritis, was sufficient to reduce the loss in weight to a considerable degree. On comparing the results obtained with ox- and sheep-brain however it is found that the former is less efficacious than the latter in this respect, although somewhat more potent in preventing polyneuritis. No explanation of this can at present be advanced.

III. *Ox-liver.*

(*Water-content 70 %.*)

TABLE III.

	Pigeon A	Pigeon 1	Pigeon 2	Pigeon B	Pigeon 3
Daily ration of natural tissue	1 gm.	2 gms.	3 gms.	4 gms.	5 gms.
Effect of diet on pigeons	Symptoms of polyneuritis on 8th day	Symptoms of polyneuritis on 47th day	Still healthy on 50th day	Still healthy on 35th day	Still healthy on 31st day
Change in weight by end of exp.	- 9 %	- 13 %	- 8 %	- 6 %	- 11 %
	Pigeon 4	Pigeon 5	Pigeon 83	Pigeon 84	Pigeon 85
Daily ration of natural tissue	7 gms.	12 gms.	3 gms.	4 gms.	5 gms.
Effect of diet on pigeons	Still healthy on 31st day	Still healthy on 31st day	Still healthy on 50th day	Still healthy on 50th day	Still healthy on 50th day
Change in weight by end of exp.	- 3 %	- 3 %	- 28 %	- 13 %	0
	Pigeon 86	Pigeon 87	Pigeon 88		
Daily ration of natural tissue	7 gms.	9 gms.	12 gms.		
Effect of diet on pigeons	Still healthy on 50th day	Still healthy on 50th day	Still healthy on 50th day		
Change in weight by end of exp.	- 12 %	- 12½ %	0		

The results indicate that 3 gms. of ox-liver daily were sufficient to prevent polyn neuritis in pigeons for 50 days, and 2 gms. delayed the development of the disease until the 47th day of the experiment. The richness of liver in the anti-neuritic substances suggested that the tissue might even be effective in curing polyn neuritis. Accordingly, minced ox-liver was dried at 30° C. by an electric fan, ground, and the dry powder emulsified with water and administered to neuritic pigeons. It was found that 7 gms. of dried liver (21 gms. liver in natural condition) rapidly cured the pigeons and prevented the reappearance of the disease for one week, while 4 gms. ameliorated the symptoms but could not effect complete recovery. Cod-liver oil in doses ranging from 2 to 8 gms. however possessed no curative properties.

IV. *Cow's milk.*

(Water-content 87 %.)

Vedder and Clark (1912) found that of four fowls fed on polished rice and 5 c.cs. of fresh cows' milk daily two developed symptoms of polyn neuritis in a short time, while the remaining two were healthy even after two months.

In the following experiments the fresh milk was well shaken in a machine immediately before use and in the case of the larger rations of milk the birds were fed four times daily.

TABLE IV.

	Pigeon 69	Pigeon 70	Pigeon 71	Pigeon 72
Daily ration of milk	2 c.cs.	3 c.cs.	4 c.cs.	6 c.cs.
Effect of diet upon birds	Severe weakness in limbs on 38th day	Symptoms of polyn neuritis on 18th day	Symptoms of polyn neuritis on 11th day	Severe weakness in limbs on 38th day
Change in weight by end of exp.	- 24 %	- 24 %	- 6 %	- 26 %
	Pigeon 74	Pigeon 57	Pigeon 52	Pigeon 53
Daily ration of milk	12 c.cs.	15 c.cs.	20 c.cs.	20 c.cs.
Effect of diet upon birds	Symptoms of polyn neuritis on 18th day	Weakness in limbs on 16th day	Symptoms of polyn neuritis on 33rd day	Symptoms of polyn neuritis on 36th day
Change in weight by end of exp.	- 12 %	- 6 %	- 12 %	- 26 %
	Pigeon 54	Pigeon 55	Pigeon 138	Pigeon 153
Daily ration of milk	35 c.cs.	35 c.cs.	10 c.cs.	30 c.cs.
Effect of diet upon birds	Symptoms of polyn neuritis on 49th day	Symptoms of polyn neuritis on 54th day	Symptoms of polyn neuritis on 11th day	Symptoms of polyn neuritis on 19th day
Change in weight by end of exp.	- 20 %	- 12 %	- 18 %	- 16 %

It is thus seen that cow's milk possessed only feeble anti-neuritic properties, as much as 35 c.c.s. daily merely delaying the development of polyneuritis until about the 50th day of the experiment. The birds receiving diets of polished rice and milk also suffered considerable loss in weight.

V. *Nuts (Husked filberts).*

(Water content 4 0/10.)

TABLE V.

	A		B	
	Pigeon 264	Pigeon 265	Pigeon 267	Pigeon 268
Daily ration of nuts	1 gm.	1 gm.	2 gms.	2 gms.
Effect of diet on pigeon	Symptoms of polyneuritis on 20th day	Symptoms of polyneuritis on 36th day	Still healthy on 50th day	Still healthy on 50th day
Change in weight by end of exp.	- 25 0/10	- 10 0/10	- 14 0/10	- 8 0/10
	C		D	
	Pigeon 269	Pigeon 270	Pigeon 271	Pigeon 272
Daily ration of nuts	3 gms.	3 gms.	5 gms.	5 gms.
Effect of diet on pigeon	Still healthy on 50th day	Still healthy on 50th day	Still healthy on 50th day	Still healthy on 50th day
Change in weight by end of exp.	0	- 11 0/10	+ 7 0/10	+ 13 0/10
	E			
	Pigeon 273	Pigeon 274		
Daily ration of nuts	7 gms.	7 gms.		
Effect of diet on pigeon	Still healthy on 27th day	Still healthy on 27th day		
Change in weight by end of exp.	+ 10 0/10	+ 8 0/10		

The results indicate that the daily addition of 2 gms. of husked filberts to the polished rice diet was sufficient to prevent polyneuritis in pigeons. For the maintenance of body-weight 5 gms. of nuts daily were necessary. As it was previously found (Cooper, 1913) that 3 gms. of lentils or 4 of husked barley were required to prevent polyneuritis, it appears that nuts are somewhat superior to these foodstuffs in anti-neuritic power.

VI. *Cheese.*

Finely-ground cheddar cheese (water-content 30%) was employed in the experiments.

TABLE VI.

	A		B	
	Pigeon 275	Pigeon 276	Pigeon 277	Pigeon 278
Daily ration of cheese	1 gm.	1 gm.	2 gms.	2 gms.
Effect of diet on pigeon	Symptoms of polynuritis on 14th day	Symptoms of polynuritis on 20th day	Symptoms of polynuritis on 31st day	Died on 38th day with symptoms of weakness.
Change in weight by end of exp.	- 8 %	- 17 %	- 10 %	- 19 %

	C		D	
	Pigeon 279	Pigeon 280	Pigeon 281	Pigeon 282
Daily ration of cheese	4 gms.	4 gms.	8 gms.	8 gms.
Effect of diet on pigeon	Symptoms of polynuritis on 22nd day	Died on 38th day with symptoms of weakness	Symptoms of polynuritis on 15th day	Died on 28th day with symptoms of weakness.
Change in weight by end of exp.	- 18 %	- 10 %	0	0

The results indicate that the addition of as much as 8 gms. of cheese daily to the polished rice diet failed to prevent polynuritis. Thus, while 35 c.c.s. of fresh milk daily (Table IV) delayed the appearance of polynuritis until the 50th day of the experiment, the equivalent amount of cheese (3.5 gms.)¹ and even more than twice this amount had no preventive effect. Possibly the anti-neuritic substances are destroyed during the process of cheese-ripening or they may be less readily absorbed from cheese than from milk.

VII. *Malt Extract.*

Experiments were carried out to ascertain whether malt extract possessed curative properties against polynuritis. Three samples of the extract were employed. Two of them were found to exert a rapid curative action upon neuritic pigeons, but a third sample even in large doses had no effect. The results of the experiments are briefly set forth below.

- Sample I (Water-content 24%)
 Minimum curing dose = 5 gms.
 „ II (Water-content 27%)
 Minimum curing dose = 7 gms.
 „ III (Water-content 18%)
 10 gms. had no curative action.

¹ 100 parts of milk yield approximately 10 parts of cheese.

Discussion of Results.

In the following table the minimum amounts of the various foodstuffs required to prevent polyneuritis in pigeons fed on polished rice are compared.

Foodstuffs	Amounts necessary to prevent polyneuritis	
	In terms of natural foodstuff	In terms of dry-weight
Ox voluntary muscle (1)	20 gms.	5.0 gms.
Ox cardiac muscle (1)	5 "	1.7 "
Ox cerebrum	6 "	1.2 "
Ox cerebellum	12 "	2.4 "
Ox liver	3 "	0.9 "
Cow's milk	> 35 "	> 3.5 "
Sheep cerebrum (1)	8 to 15 "	1.6 to 3 "
Fish voluntary muscle (1)	> 10 "	> 2 "
Egg-yolk (1)	3 "	1.5 "
Lentils (dry) (1)	—	3 "
Barley (husked) (1)	—	4 "
Nuts (husked filberts)	—	2 "
Cheese	> 8 "	> 5.6 "

(1) Cooper, 1913.

The results indicate that the various ox-tissues are not of equal anti-neuritic power, liver being most effective in preventing polyneuritis, then cardiac muscle and cerebrum, next cerebellum, and least effective, voluntary muscle and cow's milk. This order still obtains when the results are expressed in terms of dry material.

Liver is thus considerably more efficient in preventing polyneuritis than either cerebrum or cerebellum. In the natural condition cardiac muscle is about as efficient as cerebrum, but somewhat more so than cerebellum; on comparing the dried materials however heart-muscle is of smaller anti-neuritic efficacy than cerebrum, but still retains its superiority to cerebellum.

Before attempting to draw conclusions from the above results as to the actual distribution of the anti-neuritic substances amongst animal tissues and fluids, however, it is necessary to determine to what extent the various food-materials are absorbed from the alimentary canal of birds. In man egg-yolk and voluntary muscle (both of ox and fish) can be almost completely absorbed, but liver and cardiac muscle, owing to their denser structure, are less readily digested (Hutchinson, 1900). As however in my experiments these tissues were well minced, there is no reason to suppose that absorption would be less complete. Brain material, on the other hand, is imperfectly absorbed, as much as 40%

appearing in the faeces, and this may explain its relatively small efficacy in preventing polyn neuritis in birds.

I have not determined the proportion of these various foods absorbed by pigeons. It would involve a very large number of experiments to obtain results of any value, but experiments to determine the proportion absorbed when brain in various amounts is fed to birds are in progress.

The whole content of anti-neuritic substances is not absorbed even from a normal diet of grain, as it was possible to detect the presence of these substances in the excreta of a hen fed on maize, barley, and buckwheat. The active substances could also be detected in the faeces of a rabbit fed on white bread and cabbage. The excreta of the bird were collected daily for a fortnight and dried at 30° C. by an electric fan. The total amount of dry material obtained was 150 gms. This was extracted repeatedly at 35° C. with absolute alcohol in a shaking machine, the filtered extract concentrated *in vacuo* and freed from alcohol. The residue was a black gummy substance weighing 6½ gms. Three gms. of this, equivalent to one week's excretion, administered orally to a pigeon affected with polyn neuritis rapidly exerted a complete curative action and the bird again fed on polished rice remained well for a week. One gram of the extract, equivalent to two days' excretion, improved the condition of other neuritic birds, but did not effect complete recovery. The rabbit's faeces were collected daily for a week and dried at 30° C. as above. The total amount of dry material was 75 gms. This was extracted with alcohol, the procedure being similar to that employed in the case of the bird's excreta, and one half of the total amount of dry alcoholic extract, equivalent to about three days' excretion, rapidly cured a pigeon affected with polyn neuritis, but within twenty-four hours the bird again became ill.

The anti-neuritic substances present in the excreta may be derived not merely from the diet, but to some extent possibly from the bacteria growing in the large intestine. Yeast is known to be particularly rich in the active substances, so that it may be reasonably supposed that these substances are also synthesised by bacteria.

Experiments carried out to ascertain whether *B. coli* contains any considerable amount of anti-neuritic substance have been made but the extract from 2 gms. of bacteria was without effect. It is evident that the amount contained is much less than in yeast.

The results, so far, afford indications as to the composition of diets suitable for the prevention of beri-beri.

Of animal tissues heart-muscle, liver, and egg-yolk are much more valuable for this purpose than voluntary muscle of either fish or ox and, if reckoned as dry weight, are somewhat superior even to lentils, nuts, and barley, which are suitable vegetable foodstuffs to supplement the polished rice diet. The small value of flesh as a prophylactic against polyneuritis which emerges from my experiments on birds is borne out by practical experience.

According to Van Leent (1880) prior to 1876 the native crews of the Dutch East Indian Navy received a diet of polished rice (75 %) and meat or fish (25 %) and suffered considerably from beri-beri, while the European crews whose dietary contained in addition beans, peas, potatoes, and greens were almost free from the disease. Subsequently the native crews drew the same rations as the Europeans and as a result of this change there was a great fall in the number of beri-beri cases.

In 1902 and 1903¹ the native troops in the Philippines owing to the prevalence of cholera were prevented from obtaining a supply of vegetables from the markets and were consequently restricted to a diet of polished rice and meat. Soon after this change beri-beri broke out and a large proportion of the Company were affected, although for a whole year before the troops had been in excellent health.

The substitution of heart-muscle and liver for ordinary flesh in the mixed diets employed in localities where beri-beri occurs would thus be a distinct improvement, as, not only are the former tissues when properly prepared as nutritious as voluntary muscle, but they also contain higher available concentrations of the anti-neuritic substances.

SUMMARY.

1. Pigeons fed on daily rations of polished rice equal to 1/20th their initial body-weight develop symptoms of polyneuritis in about three weeks and usually lose considerably in weight.

2. The efficacies of various ox-tissues for preventing polyneuritis have been determined, and the tissues arranged according to their anti-neuritic powers are in the following descending order: liver, cardiac-muscle, cerebrum, cerebellum, voluntary muscle, and (cows') milk.

3. Alcoholic extracts of the excreta of a hen fed on unpolished grain and of the faeces of a rabbit fed on bread and cabbage cured polyneuritis in pigeons. The whole content of anti-neuritic substances

¹ Report Surg.-Gen. Army, U.S.A. 1902-1903, p. 69.

present in the dietary was therefore not absorbed or else some amount is synthesised by the bacteria of which the faeces consisted to a considerable extent. No conclusions can consequently be drawn as to the actual distribution of the active substances in the animal body, until the extent to which the various tissues are absorbed from the alimentary canal of birds has been determined.

4. Nuts (husked filberts) are very efficient in preventing polyn neuritis, being even superior to lentils and husked barley. Cheddar cheese, on the other hand, even in considerable amount, has no preventive effect.

5. Malt extract taken from two different samples readily cured polyn neuritis in pigeons. A third sample however even in large doses had no curative action.

6. For the prevention of beri-beri egg-yolk, heart-muscle, liver, nuts, barley, and lentils can be recommended as suitable foodstuffs with which to supplement the polished rice diet. As meat (voluntary muscle) has been frequently found to be ineffective in preventing epidemics of beri-beri, its replacement by heart and liver in mixed diets would be a considerable improvement, because, not only are these tissues when suitably prepared as nutritious as voluntary muscle, but they also contain the anti-neuritic substances in much higher concentration.

I desire to express my best thanks to Professor C. J. Martin, F.R.S., for valuable help and criticism.

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THE RANGE OF FLIGHT OF *MUSCA* *DOMESTICA*¹.

EXPERIMENTS CONDUCTED IN THE TOWN OF CAMBRIDGE.

Introductory note.

It having been considered desirable that further experiments upon the range of flight of flies should be carried out, the investigation of the subject was entrusted to Dr E. Hindle and Mr G. Merriman, who are engaged in research in my laboratory. Although the experiments here recorded were carried out under somewhat adverse climatic conditions, they appear of sufficient interest to warrant publication, since the results with regard to the influence of wind seem to run counter to those recorded by previous workers, and also, apart from Hewitt's work referred to below, data have hitherto been wanting in respect of the range of flight of flies (*Musca domestica* Linn.) in towns. The simple graphic method of recording the results of the flight experiments, which occurred to me during the course of the work, may commend itself to others who may carry out similar investigations in the future.

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REPORT UPON EXPERIMENTS.

BY EDWARD HINDLE, PH.D., AND GORDON MERRIMAN

(From the Quick Laboratory, Cambridge.)

(With 13 Charts.)

Previous Investigations.

THE first attempt to investigate this subject experimentally was that made by Dr Arnold, at Monsall Hospital, Manchester, in 1906 (recorded by Niven, 1906). Three hundred flies, each marked by a spot of enamel on the thorax, were liberated from the hospital and five were recovered at distances varying from 30 to 190 yards.

In 1910, Copeman, Howlett and Merriman (1911) made a number of experiments for the Local Government Board on the range of flight in open country. The investigations were carried out at Postwick (a small village about five miles east of Norwich), where the inhabitants suffered from a plague of flies, resulting from the breeding facilities afforded by a large refuse tip about half a mile away. Several thousands of chalk-powdered flies were liberated from the tip, which was situated in open country, the nearest cottages being about half a mile distant, and under these conditions invariably a number of the flies travelled distances exceeding 300 yards. In one case a fly travelled 1,700 yards, this distance being covered within 48 hours from the time of liberation. This constitutes the longest flight that has yet been recorded, although many of the flies in this series of observations were found to travel between 800 and 1,000 yards.

In these experiments, owing to the nature of the country, long distances had to be travelled before the flies could enter any houses. Therefore, the results, although important as showing the powers of flight, are no indication of the behaviour of flies in a locality where houses are many, and in consequence, food plentiful.

The same year J. S. Hine (1910), in the United States, also made an effort to determine the distance travelled by flies in open country. Three hundred and fifty flies were captured, coloured by means of spots of gold enamel, and liberated from a barn. The greatest distance at

which they were recovered was 240 yards from the point of liberation, but Hine remarks (cited by Howard, 1911, p. 55): "It appears most likely that the distance flies may travel to reach dwellings is controlled by circumstances. Almost any reasonable distance may be covered by a fly under compulsion to reach food or shelter. Where these are close at hand the insect is not compelled to go far, and consequently does not do so."

Howard (1911, p. 56) also mentions some experiments made under the direction of S. A. Forbes in Cook County, Illinois, U. S. America. Flies were trapped and, after being sprayed with a chemical solution, were then liberated from a hospital in that district. They were recovered at distances ranging up to a quarter of a mile from the point of liberation.

In the summer of 1911, Dr C. G. Hewitt (1912) carried out for the Board some experiments on this subject in Ottawa, Canada. The flies were coloured by spraying them with a solution of rosolic acid in 10 per cent. alcohol. The presence of a marked fly on a sticky fly-paper was indicated by its producing a ruddy colouration when the paper was dipped into slightly alkaline water. The flies were liberated from an island in the middle of the town and individual examples were recovered at distances of 520, 600, and 700 yards respectively. They were usually recovered in those areas of the town towards which the wind had been blowing.

As far as we are aware no other experiments upon this subject have been recorded, and it seemed desirable to obtain further information regarding the range of flight of flies in towns. The present investigation, therefore, was undertaken for the Local Government Board, in continuance of the work arranged for in 1910 (see p. 46, 3rd report on Flies as Carriers of Infection).

Experiments conducted in Cambridge during 1912.

During the months of July, August, and the first week in September, 1912, we conducted a series of experiments on the range of flight of *Musca domestica* Linn. in Cambridge. In the course of these experiments upwards of 25,000 flies have been liberated under very variable meteorological conditions, and 191 were recovered at one or other of about 50 observation stations employed for their recovery.

In all cases the flies for liberation were either caught in balloon traps or directly netted. The method of obtaining flies by breeding

was abandoned, as it was almost impossible to obtain them without numerous other species of insects, and also on account of the possible objections to such artificially-bred flies.

Prior to being liberated, the flies were kept for about 24 hours in cages made of mosquito netting and were fed on brown sugar, the moisture being supplied by a layer of damp sand. By this method it was assured that the insects had emerged sufficiently long to allow the full development of their chitinous exoskeleton, presumably necessary to obtain the full power of flight.

Preparatory to colouration, the flies were transferred from the mosquito cages into wire balloon traps. This transference was effected as follows:—the mosquito-netting cage was tied round the bottom of the balloon trap. The latter was then held towards the light and the whole of the cage surrounded with a black cloth. Owing to the strong attraction of the light, the insects all made their way towards the brightly-illuminated trap, and in passing through the small hole in the bottom of the latter, it was possible to make accurate counts of them, as not more than two or three were able to pass through at the same time. When about 1,500 flies had entered the balloon trap it was closed, then removed, and another trap fixed in its place.

The most satisfactory mode of marking the flies was found to be that devised by Nuttall (*vide* Jepson, 1909), and this was employed in all our experiments. The balloon trap containing the flies was placed in a large brown-paper bag, in which was a handful of powdered black-board chalk, coloured either red, orange, or yellow. The mouth of the bag was then closed, and the whole gently shaken for one or two minutes so that the flies were thoroughly dusted with the chalk. The balloon was then removed and after being taken to the point selected for the liberation, the trap was opened and the flies allowed to escape in any direction they chose. The flies were recovered either by means of fly-papers or balloon traps, several of which were exposed at the various observation stations. The traps and papers were examined for several successive days after the liberation of a number of coloured flies, and as the observation stations extended as far as 900 yards from the point of liberation, comprising both thickly and sparsely populated localities, an accurate idea of their distribution was thus obtained. Full meteorological data were kindly supplied by Messrs W. E. Pain, chemists, Sidney Street, Cambridge. Their observations were made in the centre of the town and in consequence indicated the exact meteorological conditions under which the flies travelled. In all, thirteen

experiments were completed, after which the investigations had to be concluded as a result of the bad weather.

When the flies were liberated in the morning, the traps and fly-papers were examined on the afternoon of the same day. Subsequently, however, the observation stations were visited every morning and, therefore, any flies then recovered would have been exposed mostly to the winds of the preceding days. This point should be remembered in examining the following results, for in some cases the flies seem to have travelled with the wind owing to its change of direction on the day of recovery.

Owing to the variable meteorological conditions, we consider it advisable to describe each of these experiments separately.

Experiment 1.—16th–21st July, 1912.

About 1,000 orange-coloured flies were liberated from the roof of the Medical Schools¹ at 11 a.m. on July 16th. The weather was warm and a light wind was blowing from the east, the conditions thus being favourable for the distribution of the flies. In spite of this, as will be seen from the following chart, only five examples were recovered, and these at comparatively short distances. However, since all the fly-papers and traps were set in buildings, the fine and warm weather prevailing at the time of the experiment would explain the few recoveries of marked flies, as cold and damp appear to be the main factors which cause these insects to seek shelter in houses. (See Chart 1.)

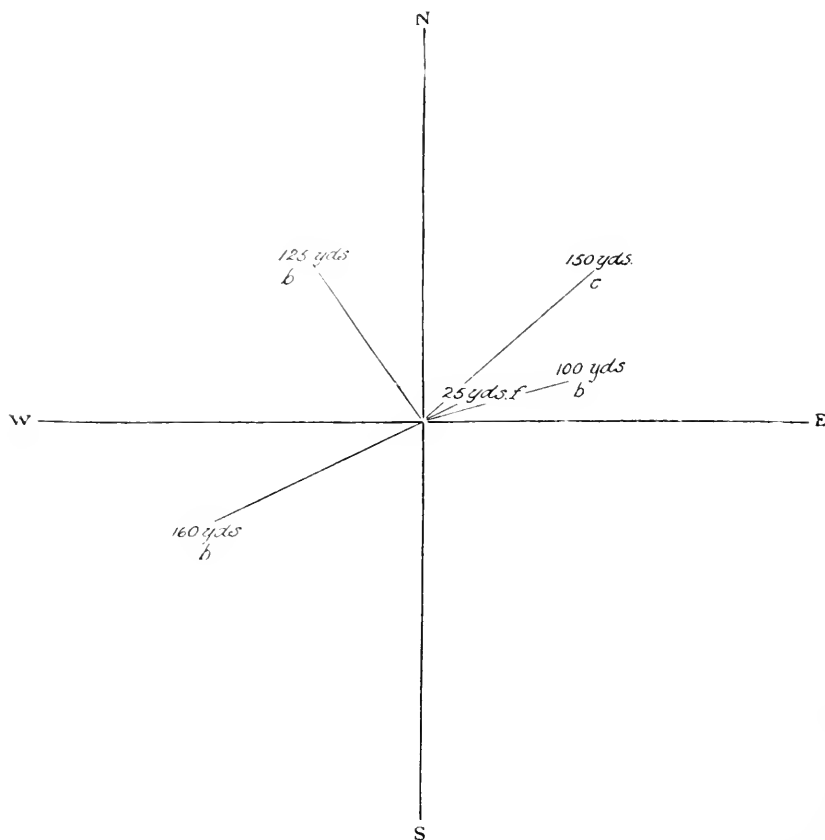
It will be noticed that of the five flies caught, all took a direction either across the wind, or somewhat against it. For example, on July 17th flies were recovered from three stations situated respectively E.N.E., N.W. by N., and W.S.W. These were recovered subsequent to the first day, none of them at a greater distance than 160 yards.

Experiment 2.—20th–23rd July, 1912.

1,650 red-coloured flies were liberated from the roof of the Medical Schools on July 20th. 1,050 were set loose at 10.30 a.m., and the remaining 600 at 1.15 p.m. The weather was warm, but later in the day was inclined to be showery, and probably owing to the latter fact a large number of flies were recovered at distances ranging up to as much as 650 yards. As will be seen from the accompanying chart (2), the

¹ A height of 45 feet.

conditions prevailing throughout this experiment seem to be very favourable for the distribution of the flies, as 21 were re-caught. Here again, in all the longer flights the flies tended either to fly across or

Chart 1¹.

100 yds. = 0.75 inch.

	Date	Direction of wind	Velocity of wind in miles per hour	Thermometer		Rainfall in inches
				Maximum	Minimum	
<i>a</i>	July 16	E.	6	89	59	0.0
<i>b</i>	.. 17	N.	8	89	58	0.0
<i>c</i>	.. 18	NNW.	7	78	57	0.0
<i>d</i>	.. 19	NW.	8	68	48	0.0
<i>e</i>	.. 20	N.	6	59	50	0.26
<i>f</i>	.. 21	SE.	3	68	56	0.02

¹ The letters at the points at which the marked flies were captured represent the day on which they were caught, *e.g.*, *a* = 1st day; *b* = 2nd day, and so on; *aa* would thus signify that two marked flies were caught on that day. The figures represent the distance from the point of liberation.

against the wind. For example, the individual that flew 440 yards was recovered from a station W.S.W. from the point of liberation, and that at 650 yards from a point S.E. by S., whilst in both cases the wind on the day of recovery and preceding day was S.W. and S.E. respectively. It should, however, be mentioned that three flies recovered at a distance of 275 yards had apparently flown directly down the wind, which was from the S.E. on the previous day.

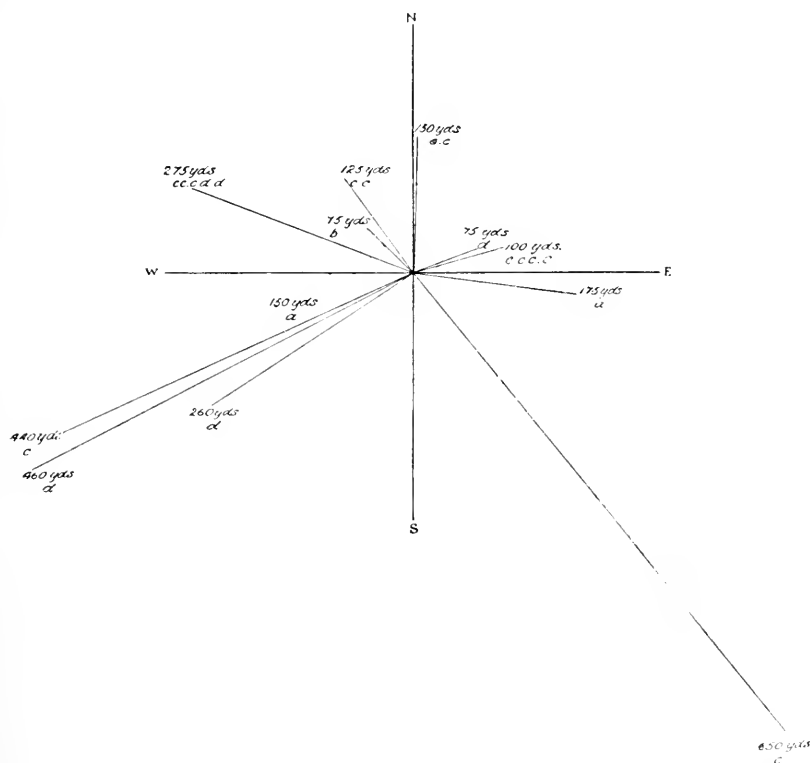


Chart 2.

100 yds. = 0.48 inch.

	Date	Direction of wind	Velocity of wind in miles per hour	Thermometer		Rainfall in inches
				Maximum	Minimum	
a	July 20	N.	6	59	50	0.26
b	„ 21	SE.	3	68	56	0.02
c	„ 22	SW.	2	75	56	0.0
d	„ 23	SE.	3	70	59	0.02

Experiment 3.—23rd–27th July, 1912.

1,350 yellow-coloured flies were liberated from the roof of the Medical Schools at 9.30 a.m. on July 23rd. The weather was warm and showery, with a slight S.E. wind blowing. Although the sun was shining at the time of liberation, a slight shower coming shortly afterwards drove a number of the flies (20–30) into the Quick Laboratory, which is situated on the ground floor of the Medical Schools. Possibly on account of this shower, a comparatively small number of flies were recovered at any of the stations, as they were mostly driven into buildings close at hand, for large numbers of the flies were observed to take shelter in the sheds below the point of liberation. One fly was recovered at a distance of 440 yards, to reach which, if it flew in a straight line, it had to pass over three high buildings. Yet again, the tendency of flies to travel *across* the wind was noticeable, as in this case the above-mentioned fly travelled in a S.S.E. direction, whilst the wind on the preceding day was W.S.W.

Experiment 4.—26th–29th July, 1912.

1,500 red-coloured flies were liberated from the roof of the Medical Schools at 9.30 a.m. on July 26th. The weather was warm and fine, and a moderate breeze was blowing. The fly which travelled the longest distance (400 yards) would appear, from the chart, to have travelled *with* the wind, and so constitute an exception to our rule, but as the fly was caught early on the morning of the 27th it had, therefore, mainly been subjected to the wind of the previous day. In this case it travelled in a N. by W. direction, whilst the wind of the previous day was W.S.W. We would also call attention to the fact that this entailed passing through the most thickly-housed part of Cambridge. One of our most striking instances of flies travelling deliberately *against* the wind is that afforded by an individual which was found in a trap 325 yards distant in a W. by S. direction, only 1½ hours after the time of liberation, although the wind was blowing steadily the whole time five miles an hour in a W.S.W. direction. (See Chart 4.)

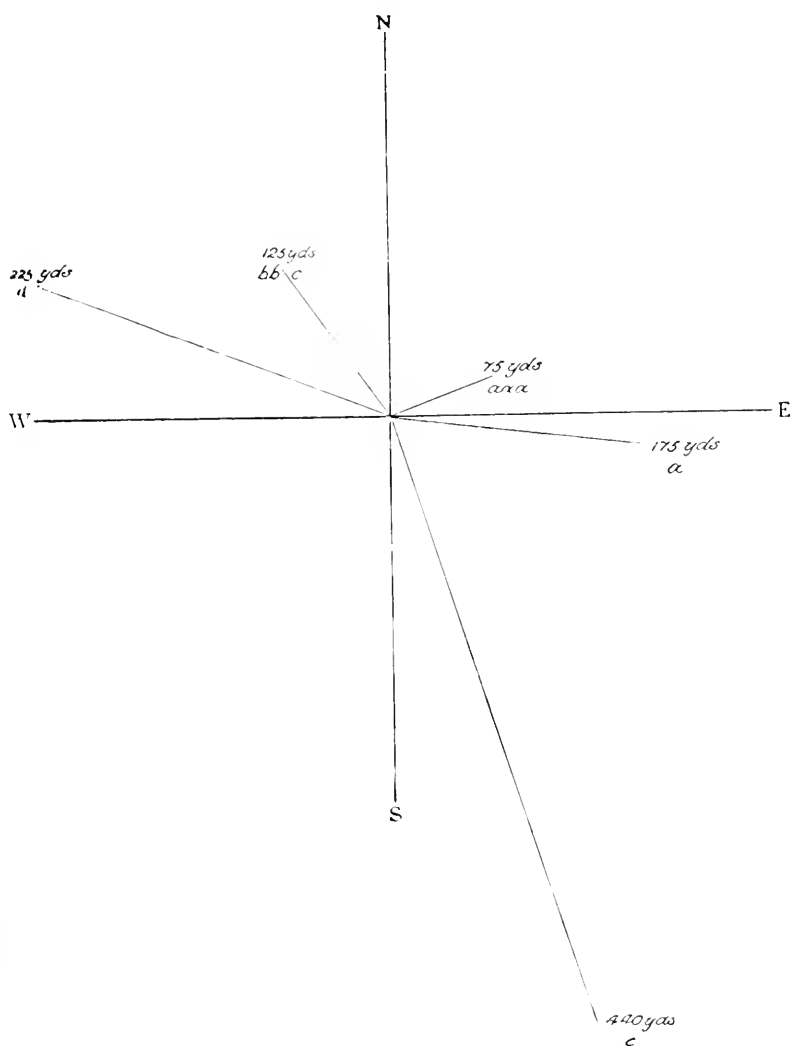


Chart 3.

100 yds. = 0.75 inch.

	Date	Direction of wind	Velocity of wind in miles per hour	Thermometer		Rainfall in inches
				Maximum	Minimum	
<i>a</i>	July 23	SE.	3	70	59	0.02
<i>b</i>	„ 24	SSE.	5	75	60	0.01
<i>c</i>	„ 25	S.	3	79	63	0.07
<i>d</i>	„ 26	WSW.	5	79	55	0.0
<i>e</i>	„ 27	SE.	4	76	62	0.13

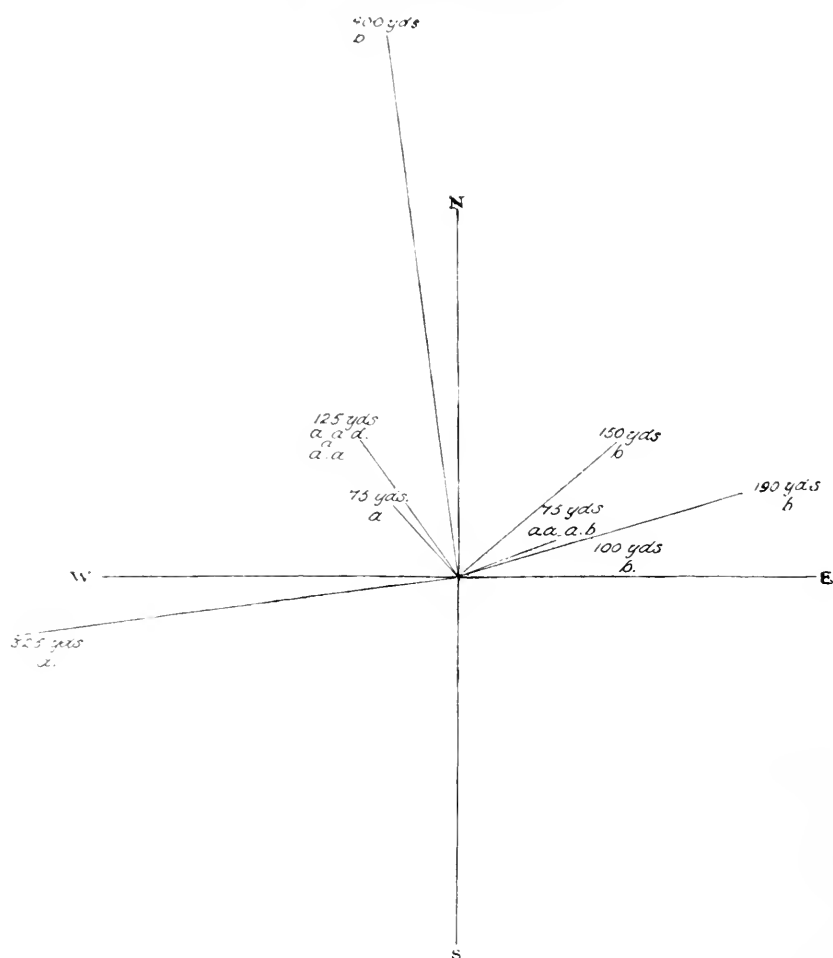


Chart 4.

100 yds. = 0.7 inch.

	Date	Direction of wind	Velocity of wind in miles per hour	Thermometer		Rainfall in inches
				Maximum	Minimum	
a	July 26	WSW.	5	79	55	0.0
b	.. 27	SE.	4	76	62	0.13
c	.. 28	SW.	8	77	61	0.01
d	.. 29	SSW.	12	71	56	0.06

Experiment 5.—29th July–1st August, 1912.

1,500 orange-coloured flies were liberated at 11.15 a.m. on July 29th. In all our previous experiments the flies were liberated at an altitude of 45 feet, but in this case they were set free on the ground. The temperature was somewhat lower than on the previous occasions, and it

was raining slightly at the time of liberation, so that it is not surprising that only six flies were recovered. In spite of the strong wind (12 miles per hour) that was blowing throughout the whole period of the experiment, the two flies that travelled the longest distance were both recovered at a point S.W. by W., whilst the wind was from S.S.W. to W. (See Chart 5.)

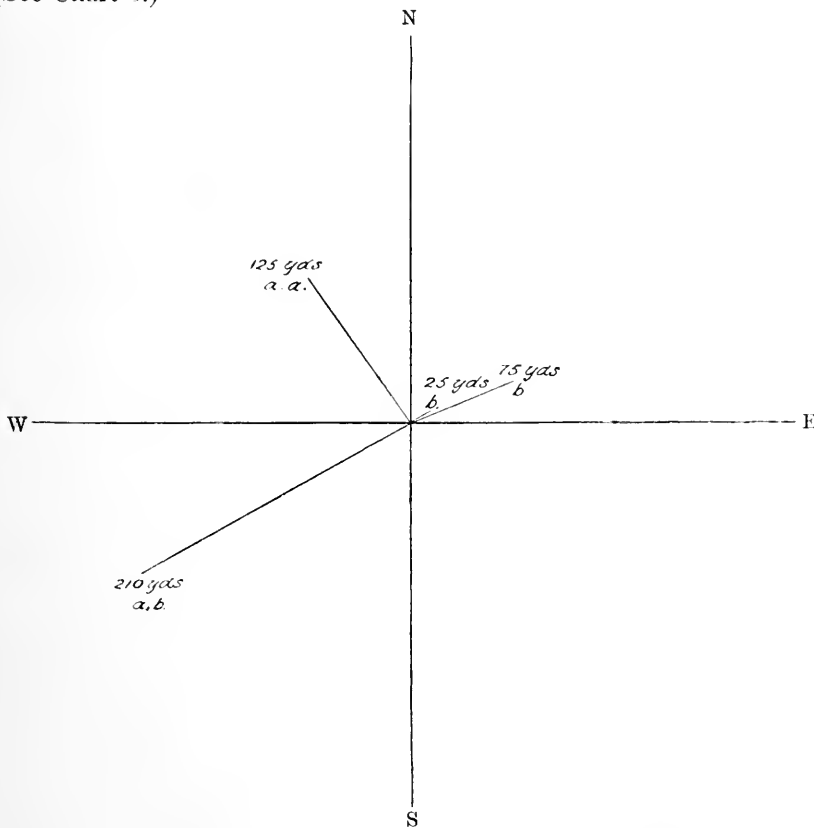


Chart 5.

100 yds. = 0.75 inch.

	Date	Direction of wind	Velocity of wind in miles per hour	Thermometer		Rainfall in inches
				Maximum	Minimum	
<i>a</i>	July 29	SSW.	12	71	56	0.06
<i>b</i>	" 30	W.	12	65	53	0.08
<i>c</i>	" 31	S.	8	69	52	0.0

Experiment 6.—6th–12th August, 1912.

2,400 red-coloured flies were liberated from the ground at 11.30 a.m. on August 6th. A strong wind (11 miles per hour) was blowing at the time of liberation, and several showers fell during the day, but,

nevertheless, no less than 34 flies were recovered at distances ranging up to 325 yards from the point of liberation. It will be noticed that most of the 15 flies which travelled a distance of more than 150 yards had flown either across, or in the teeth of, the wind prevailing on the day previous to their recovery. In this case, a number of flies were recovered from rooms at an altitude of 30 feet, and in many cases the insects must have flown over buildings at least 50 feet high.

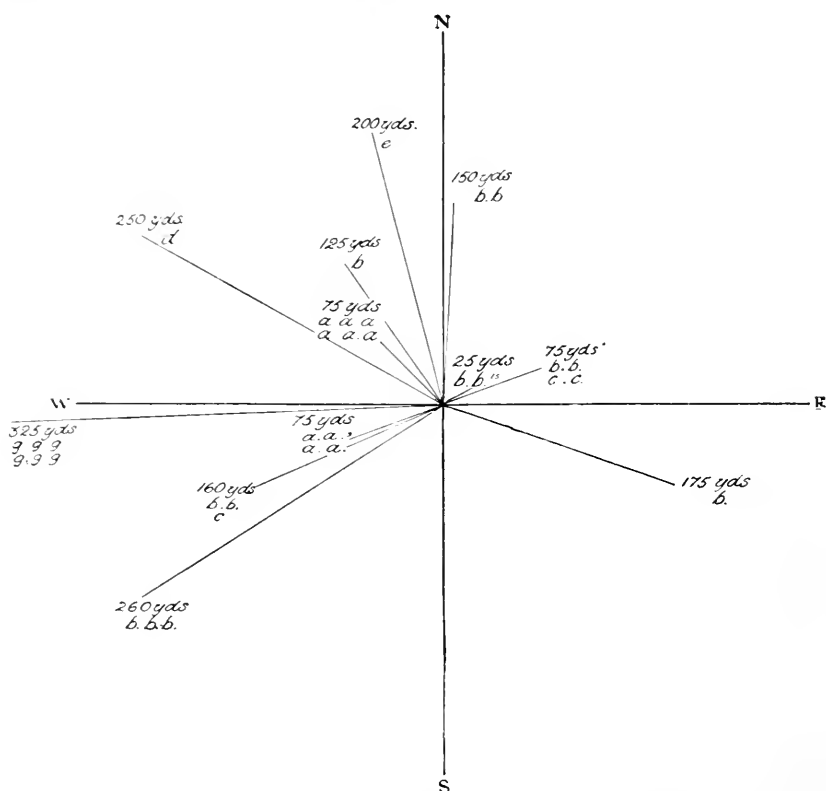


Chart 6.

100 yds. = 0.7 inch.

	Date	Direction of wind	Velocity of wind in miles per hour	Thermometer		Rainfall in inches
				Maximum	Minimum	
a	Aug. 6	SE.	11	68	56	0.1
b	„ 7	WSW.	10	65	53	0.2
c	„ 8	W.	8	66	52	0.16
d	„ 9	W.	6	67	54	0.04
e	„ 10	S.	5	67	52	0.0
f	„ 11	W.	6	66	49	0.08
g	„ 12	E.	4	64	49	0.03

Experiment 7.—9th–12th August, 1912.

3,000 yellow-coloured flies were liberated from the ground on the 9th of August at 11 a.m. The weather from now onwards was cold and

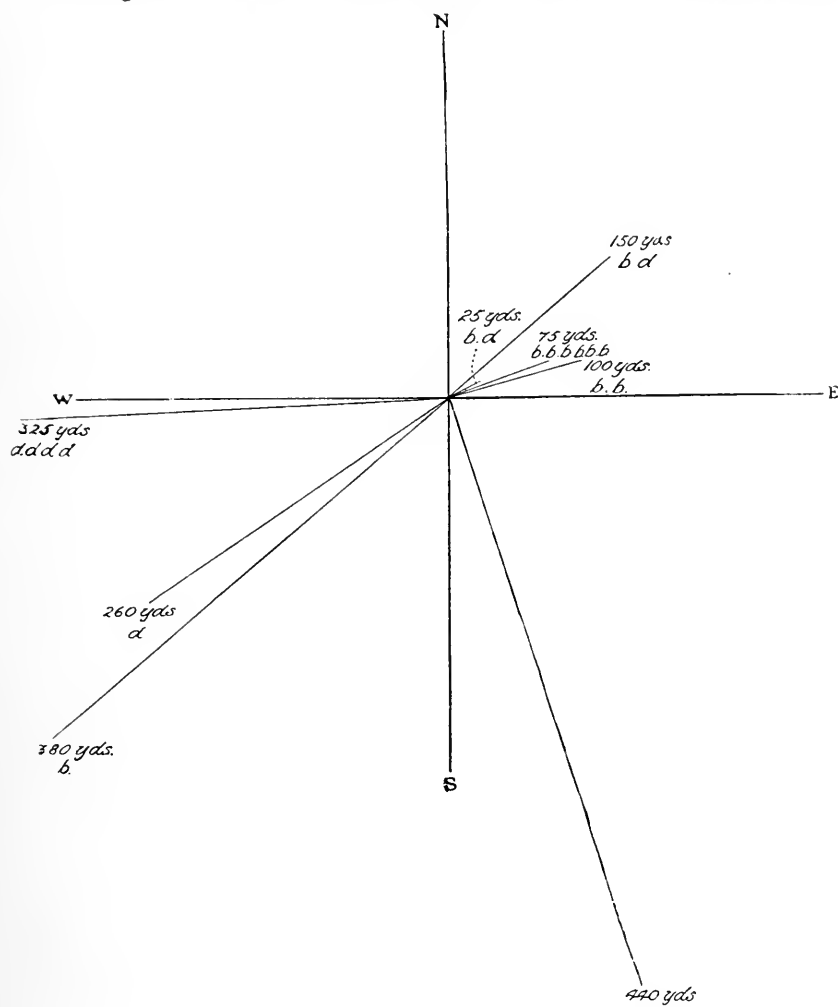


Chart 7.

100 yds. = 0.73 inch.

	Date	Direction of wind	Velocity of wind in miles per hour	Thermometer		Rainfall in inches
				Maximum	Minimum	
a	Aug. 9	W.	6	67	54	0.04
b	" 10	S.	5	67	52	0.0
c	" 11	W.	6	66	49	0.08
d	" 12	E.	4	64	49	0.03

rainy, so that the conditions were somewhat unfavourable for the distribution of the flies. In this experiment, however, 19 flies were recovered at stations up to a quarter of a mile distant in directions either across or against the wind.

Experiment 8.—12th–17th August, 1912.

1,500 red-coloured flies were liberated from the ground on August 12th at 4 p.m., since we were desirous of finding out whether the hour

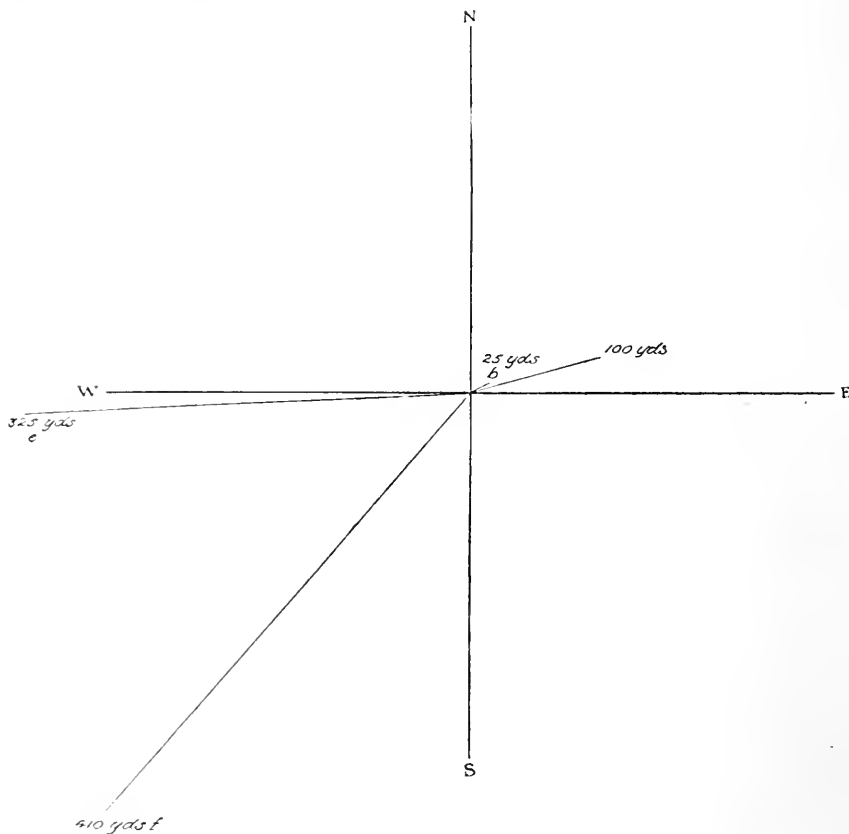


Chart 8.

100 yds. = 0·7 inch.

	Date	Direction of wind	Velocity of wind in miles per hour	Thermometer		Rainfall in inches
				Maximum	Minimum	
<i>a</i>	Aug. 12	E.	4	64	49	0·03
<i>b</i>	" 13	N.	7	67	45	0·05
<i>c</i>	" 14	W.	7	61	46	0·0
<i>d</i>	" 15	W.	8	61	51	0·03
<i>e</i>	" 16	W.	7	61	51	0·05
<i>f</i>	" 17	WSW.	9	67	56	0·05

of liberation had any effect on the distribution of the flies. Considering that in this experiment only four flies were recovered, it is probable that flies liberated in the afternoon do not disperse so readily as those set free during the morning. During the night following the day of liberation the thermometer fell to 45° F., and the succeeding days being cold, the flies having taken shelter probably remained under cover. This view is supported by the fact that the two flies which travelled any distance were not recovered till the 4th and 5th days respectively. These two flies had both travelled against the wind, which was blowing from seven to nine miles per hour.

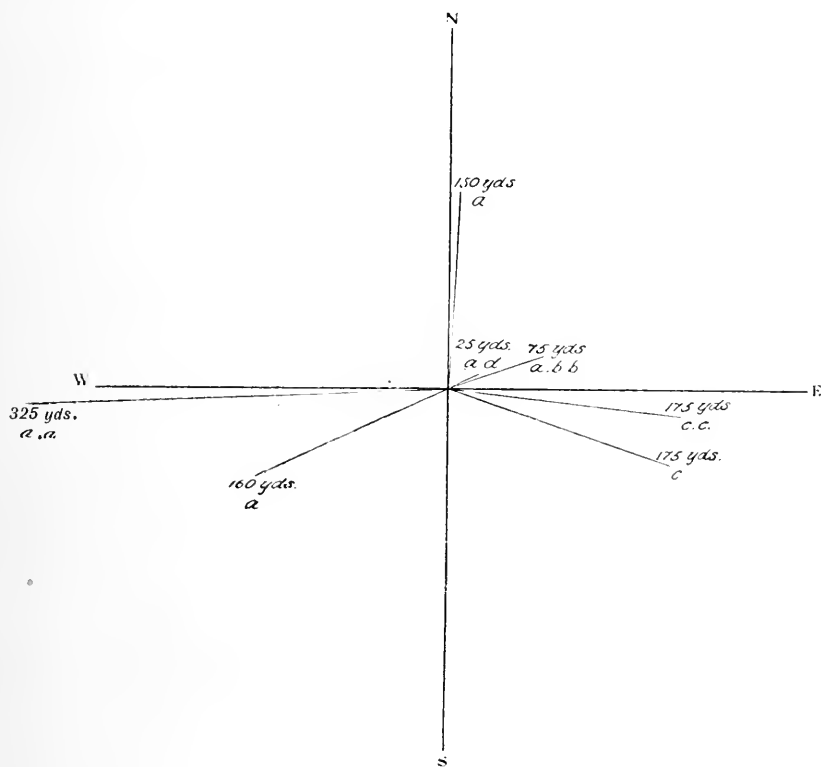


Chart 9.

100 yds. = 0.7 inch.

	Date	Direction of wind	Velocity of wind in miles per hour	Thermometer		Rainfall in inches
				Maximum	Minimum	
<i>a</i>	Aug. 16	W.	7	61	51	0.05
<i>b</i>	„ 17	WSW.	9	67	56	0.05
<i>c</i>	„ 18	W.	8	68	57	0.06
<i>d</i>	„ 19	S.	7	68	53	0.35

Experiment 9.—16th–19th August, 1912.

1,500 orange-coloured flies were liberated from the ground on August 16th at 10 a.m. The weather was cold and wet, but, nevertheless, on the same day two flies were recovered at a distance of 325 yards, five hours after they were liberated. During this period a west wind of seven miles per hour had been blowing, and the point at which the flies were recaptured (a butcher's shop) was W. by S. It seems possible that they were attracted in this direction by the smell of meat coming down the wind. (See Chart 9.)

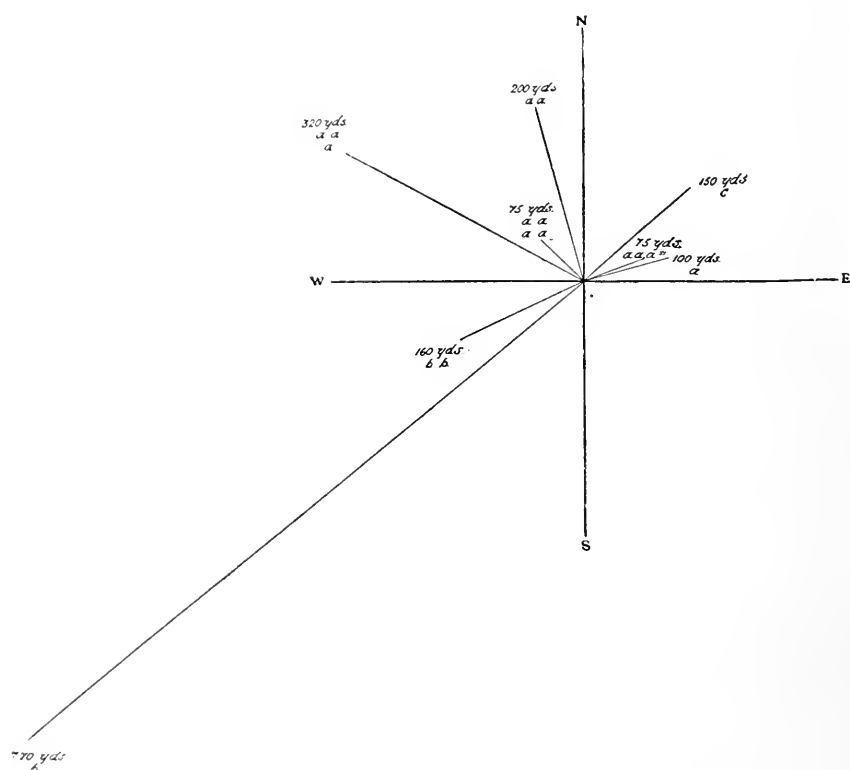


Chart 10 (A).

100 yds. = 0·5 inch.

	Date	Direction of wind	Velocity of wind in miles per hour	Thermometer		Rainfall in inches
				Maximum	Minimum	
<i>a</i>	Aug. 31	NW.	8	68	49	0·01
<i>b</i>	Sept. 1	W.	6	65	52	0·22
<i>c</i>	.. 2	NW.	6	66	53	0·15

Experiment 10 (A and B).—31st August–2nd September, 1912.

In this case 4,000 flies were liberated at 10 a.m. on August 31st: 2,000 yellow-coloured flies were liberated from the ground, and at the same time 2,000 red-coloured flies were set free from the roof, the object being to determine whether the altitude at which the flies were set free had any effect on their dispersal.

The difference between the two cases was not very marked, but the flies liberated on the roof tended to become more widely diffused than

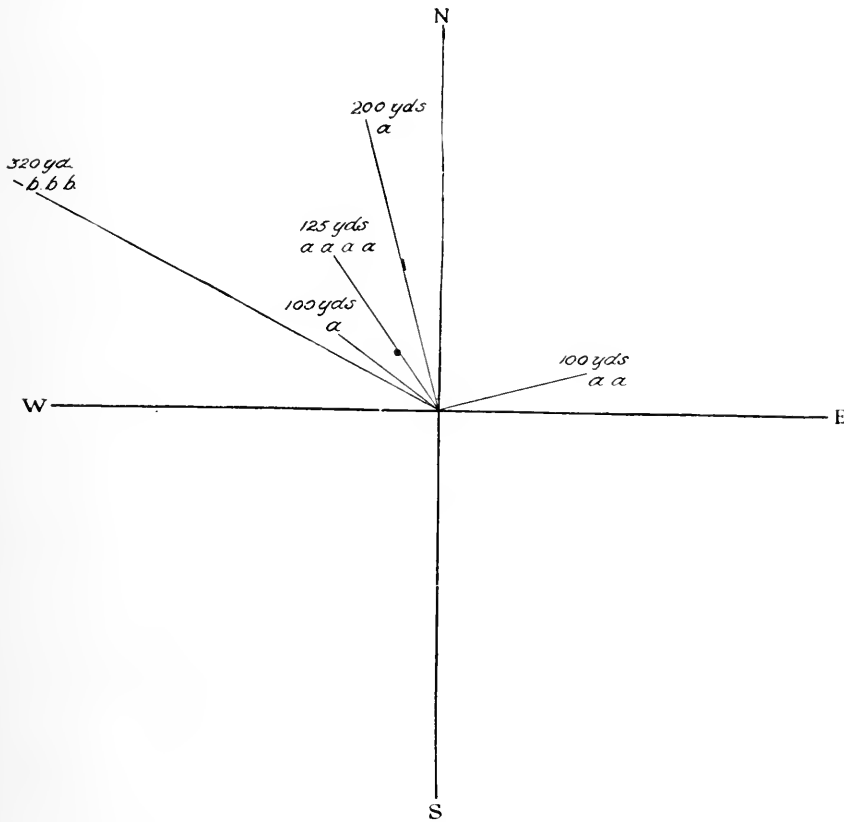


Chart 10 (B).

100 yds. = 0.8 inch.

	Date	Direction of wind	Velocity of wind in miles per hour	Thermometer		Rainfall in inches
				Maximum	Minimum	
a	Aug. 31	NW.	8	68	49	0.01
b	Sept. 1	W.	6	65	52	0.22
c	,, 2	NW.	6	66	53	0.15

those set free at ground level. Of those set free from the roof, 17 were recovered at distances ranging up to 770 yards, and of those liberated on the ground 11 at distances up to 320 yards.

A. 2,000 *red-coloured flies liberated from roof, August 31.*—In spite of the unfavourable weather, which was both cold and wet, one of these flies was recovered at a distance of 770 yards, which constitutes the longest flight which we have observed throughout this investigation. It should be noted, however, that of this 770 yards, 250 were across open fen-land. This was the first occasion on which we had a predominating north-westerly wind during an experiment, and also the first time flies were recovered from any considerable distance at stations N.W. of the point of liberation. Three flies were recovered at a restaurant situated N.W. by W. and 320 yards distant, and as the wind had been blowing from this direction, it is possible that they were attracted by smell. To reach this point it would be necessary for the flies to traverse a very thickly-housed locality comprising many high buildings.

B. 2,000 *yellow-coloured flies liberated from ground, August 31.*—As mentioned above, these flies did not travel quite as far as those liberated from the roof, but three were recovered from the same restaurant—a distance of 320 yards. In every case, the flies recovered took a northerly or north-westerly direction, the prevailing wind being N.W.

Experiment 11 (A and B).—5th–7th September, 1912.

6,000 flies were liberated at 11 a.m. on September 5th. A strong wind (11 to 12 miles per hour) was blowing, and the weather was very cold. As in the previous experiment (10) half the flies were liberated from the ground and half from the roof. This experiment was very unsatisfactory, as the cold wind rendered the flies very torpid; but, as before, a larger number of those liberated from the roof were recovered.

A. 3,000 *orange-coloured flies liberated from roof, September 5.*—In this case, 23 flies were recovered at distances up to 190 yards, and no less than 17 from one station 125 yards distant. The short distances traversed clearly demonstrate the effect of cold in rendering these insects torpid.

B. 3,000 *red-coloured flies liberated from the ground, September 5.*—In this experiment only 14 flies were recovered, as against 23 in (A); but it is rather curious that two of these flies covered greater distances

than any of those liberated from the roof. This, however, is insignificant, as the maximum distance travelled was only 210 yards.

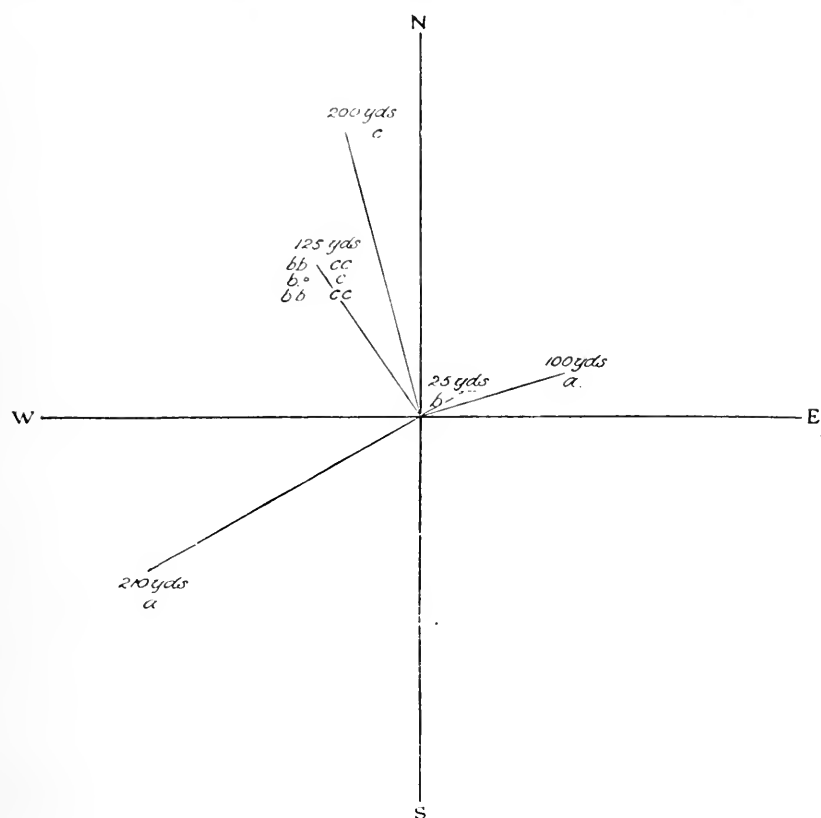


Chart 11 (A).

100 yds. = 0.75 inch.

	Date	Direction of wind	Velocity of wind in miles per hour	Thermometer		Rainfall in inches
				Maximum	Minimum	
a	Sept. 5	W.	11	66	49	0.0
b	„ 6	W.	12	61	45	0.02
c	„ 7	NNW.	11	59	45	0.0

Discussion of the above-described Experiments.

Unfortunately, nearly all our experiments in Cambridge were seriously handicapped by the great difficulty of obtaining flies in sufficient numbers and also by the adverse meteorological conditions. Throughout August the weather was so bad that from the 19th to the

31st of this month not a single fly could be liberated. During the early part of September nearly all the flies became infected with *Empusa muscae*, and this, in conjunction with the cold weather, brought the investigation to a sudden end. In the earlier experiments we should have preferred to have liberated at least double the number of flies, but owing to the difficulty of procuring them this was impossible. Our results, therefore, are not as complete as we could have wished.

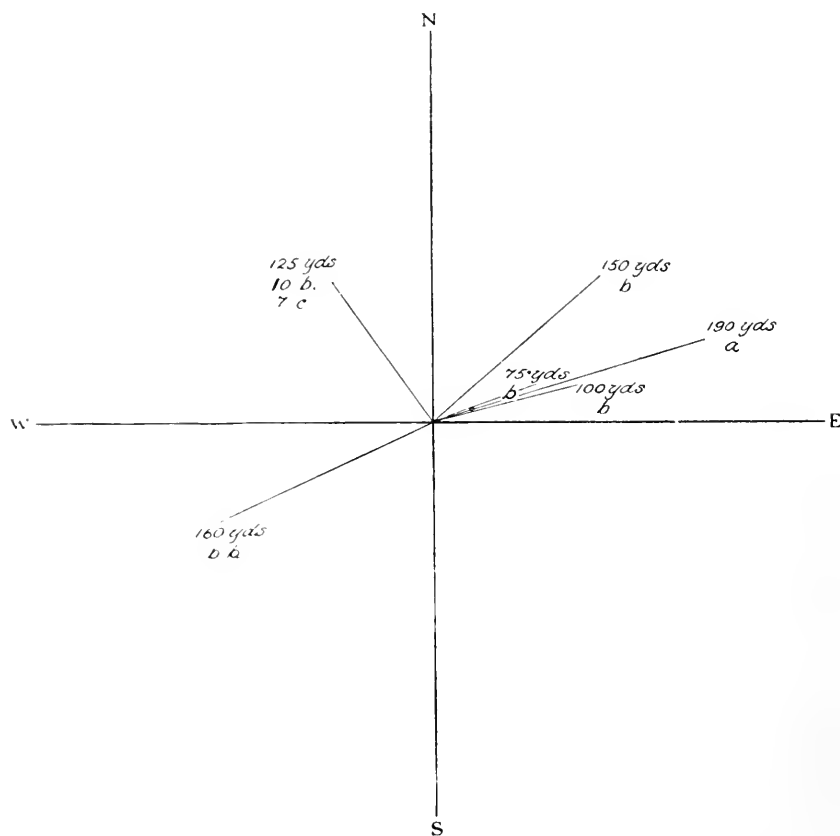


Chart 11 (B).

100 yds. = 0.77 inch.

	Date	Direction of wind	Velocity of wind in miles per hour	Thermometer		Rainfall in inches
				Maximum	Minimum	
a	Sept. 5	W.	11	66	49	0.0
b	.. 6	W.	12	61	45	0.02
c	.. 7	NNW.	11	59	45	0.0

Nevertheless, owing chiefly to the large number of stations employed for the recovery of the flies and their being situated in various directions, we have been able to obtain certain definite results.

The most striking feature brought out by our experiments is the marked effect of the direction of the wind on the courses taken by the flies. After a careful examination of all our results, it appears to us that the flies tended to travel either directly *against* or *across* the wind. The main exceptions to this rule were those recovered within a radius of about 150 to 200 yards from the point of liberation, and probably these flies were individuals that had merely selected the first shelter they could find. These results differ somewhat from those of Copeman, Howlett, and Merriman (1911), who found that for the most part the flies tended to travel *with* the wind. But it should be remembered that not only were these investigators working in open country, but also their traps were necessarily set at stations to the east of the point of liberation, and consequently none of the flies that flew in a westerly direction would be recovered.

Owing to lack of opportunities we have been unable to decide why, in our experiments, the flies tended to travel either against or across the wind. Two explanations are possible:—

(1) The flies may tend to fly against any current of air to which they are subjected. This property is known as positive anemotropism, and is possessed by some other insects and birds. In view, however, of the results obtained by Copeman, Howlett, and Merriman (1911), we cannot come to definite conclusions on this point, and further experiments are required to determine if other factors than wind-direction influence the direction of flight.

(2) The flies may travel against the wind, being attracted by any odours it may convey from a source of food. A point in favour of this supposition is the nature of the stations at which flies were recovered after they had travelled any distance. These comprised a butcher's shop, public houses, and a restaurant, all of which gave off odours that are notoriously attractive to flies.

The maximum distance travelled by any of the flies we liberated in Cambridge was 770 yards, which is considerably less than that covered by those liberated in the open country at Postwick—in one case as much as 1,700 yards. This difference may be attributed to the absence of shelter in the case of the Postwick flies, whereas in Cambridge food and shelter were always plentiful. On the whole, we do not think it likely that, as a rule, flies travel more than a quarter of a mile in thickly-

housed areas. Throughout our experiments only two flies exceeded this distance and in the case of the individual that had travelled 770 yards, a large part of its journey was across open fen-land.

The chief factors influencing the dispersal of the flies are probably the temperature, weather, and the time of day when the insects are liberated. The effect of temperature is very marked, as when it is low the flies become torpid and seek the first available shelter. This is shown in Exps. 11 (A) and 11 (B), respectively. Fine weather is also a necessary condition for long flights, as rain at once drives the flies into shelter. The ideal experimental conditions for a flight experiment are two or three days of fine warm weather, during which the flies can make their flight, succeeded by a wet or showery day, when they are driven indoors, and thus can be recorded at the various stations.

With regard to the altitude of the point of liberation, flies set free from the roof tended to disperse slightly better than those liberated from the ground, but the differences are not very considerable.

With regard to the vertical flight of the house-fly, although we have found no means of estimating the maximum, nevertheless, during our experiments, when liberating them from the ground, we have frequently observed the flies at once mount almost vertically upwards to a known height of 45 feet.

SUMMARY.

Under the conditions of our experiments, indication was afforded that—

(1) House-flies tend to travel either *against* or *across* the wind. The actual direction followed may be determined either directly by the action of the wind, or indirectly owing to the flies being attracted by any odours it may convey from a source of food.

(2) It is likely that the chief conditions favouring the dispersal of flies are fine weather and a warm temperature. The nature of the locality is another considerable factor, as in towns flies do not travel as far as in open country, this being probably due to the food and shelter afforded by the houses.

(3) Under experimental conditions, the height at which the flies are liberated, and also the time of day, appear to influence the dispersal of the insects. As judged by one experiment, when flies are set free in the afternoon they do not scatter so well as when liberated in the morning.

(4) The maximum flight in thickly-housed localities in our experiments was about a quarter of a mile; but in one case a single fly was recovered at a distance of 770 yards. It should be noted, however, that part of this distance was across open fen-land.

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NOTE ON THE COLOUR-PREFERENCE OF FLIES¹.

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IN the course of our investigations on the biology of flies, during the year 1912, a certain number of experiments were performed in order to ascertain whether house-flies possess any colour preference. In the case of mosquitoes, Nuttall (1901) has shown that these insects have a very well-defined preference for certain colours. When a number of boxes, lined with different coloured materials, were placed in a tent containing mosquitoes, the latter occurred most frequently on navy-blue, and, in descending order, on dark red, brown, scarlet, black, slate-grey, dark green, violet, leaf-green, blue, pearl-grey, pale green, light blue, ochre, white, orange, and yellow. Very few insects indeed were found to rest on the last seven colours.

A French observer, Fé, published the statement that, having noticed that flies did not rest upon walls covered with blue paper, he blue-washed the walls of his milk houses and found that insects did not visit them. Galli-Valerio (1910), who refers to Fé's statement, conducted a few experiments on this subject.

Different coloured pieces of paper of equal size were pasted over the walls of a large glass box, and afterwards a number of house-flies were introduced. The position of the cage was changed several times, and in each position the number of flies resting on the respective colours was carefully counted. As a result the flies were found to rest on each colour in the following numbers:—Clear green, 18; rose, 17; clear yellow, 14; azure, 13; clear red, 10; dark grey, 9; white, 9; dark red, 8; black, 7; pale grey, 5; dark yellow, 5; dark green, 5; red, 4; orange, 3; clear brown, 3; pale rose, 3; very clear green, 2; blue, 1; pale violet, 1; dark brown, 1; lemon yellow, 1.

Galli-Valerio notes that 87 flies rested on clear colours and 52 on the dark ones. From these results it seems that, although very few

¹ Reprinted from the *Reports to the Local Government Board on Public Health and Medical Subjects* (1913, New Series, No. 85, pp. 20-41, with the permission of His Majesty's Stationery Office).

flies settled on the blue, the closely-related colour, azure, was one of those the most visited.

These results seemed so uncertain that it was decided to perform some experiments on the subject.

A strip of cardboard, 24 inches long and 7 inches wide, was painted with bands of colours 4 inches wide. The whole was then covered with a sticky substance so that any flies which settled on the card were caught. Later, it was found more convenient to cover the front of the coloured strip with transparent sticky paper, through which the colours were quite visible. The relative positions of the bands of colour were changed from day to day, and the flies that had accumulated on them removed and counted.

The results were as follows; the figures indicating the number of flies caught on each colour.

Date	Blue	Yellow	Black	Red	White	Green
20. VII. 12	24	47	21	37	41	55
22. VII. 12	8	8	5	9	29	30
23. VII. 12	33	34	16	23	28	27
24. VII. 12	16	20	12	17	9	10
25. VII. 12	22	19	20	20	22	24
26. VII. 12	17	23	10	15	23	25
27. VII. 12	17	16	21	17	13	14
29. VII. 12	25	25	22	26	10	12
30. VII. 12	8	14	18	16	9	10
31. VII. 12	13	13	14	8	5	7
1. VIII. 12	11	17	15	15	25	36
2. VIII. 12	12	22	22	18	16	19
3. VIII. 12	18	13	19	21	10	4
Total	224	271	215	242	240	273

A comparison of the total number of flies collected on each colour clearly shows that, under the conditions of the experiment, flies do not display any marked colour preference. Therefore, it seems unlikely that the adoption of any particular colour for the walls of houses and stables will have any effect on the numbers of flies entering them.

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AN ELECTRO-CHEMICAL APPARATUS FOR THE DISINFECTION AND CLEANSING OF CULTURES AND SLIDES FOR USE IN BACTERIOLOGICAL AND PATHOLOGICAL LABORATORIES.

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*(From the Bacteriological Department of the City and
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(With Plate II and 1 Text-figure.)

It is the custom in most laboratories where infected material is used during the progress of experimental work to have receptacles containing a disinfectant of some kind, placed so that the worker may drop any small piece of apparatus or culture into it which he has finished with, in order that such material should not be a source of danger to himself and to others in the laboratory. The disinfecting agent is more often than not some saponified tar-acid product, which, although lethal to naked bacteria may, or may not, destroy infection under the circumstances in which it is used. The fluid is also somewhat costly, apart from being uncertain in its action when resistant spores are being dealt with.

During an experimental enquiry¹ in relation to the sterilization of bacteria, the author made several tests with sodium and calcium hypochlorite which are well known to be powerful disinfectants even in very dilute solution. Instead, however, of adding the hypochlorite to

¹ Beattie, J. M. and Lewis, F. C. (1913). The utilisation of electricity in the continuous sterilization of milk. *Journ. Path. and Bact.* xviii. July 1913, pp. 120-122.

the fluid to be sterilized, it was decided to produce the disinfectant in the fluid by the aid of electricity.

When sodium chloride is dissolved in water, and a unidirectional electric current of suitable strength is allowed to flow through the solution, the salt is decomposed into sodium and chlorine, the water being electrolysed into hydrogen and oxygen. If metal electrodes are used, a chloride of the metal is produced. In the event, however, of the electrodes being of neutral material such as carbon, some of the chlorine is evolved free and under suitable conditions sodium hypochlorite is produced by the combination of the chlorine with sodium hydroxide. In order to ensure the formation of the hypochlorite, the temperature should not exceed 50° C. The temperature of the fluid can be regulated by directly varying the strength of the electric current used, or by modifying the internal resistance of the cell by altering the distance between the electrodes.

If a mechanical device be used to bring about more efficient mixing of the products of electrolysis or, when the direction of the current is reversed, say, every five seconds, a better result is obtained, *i.e.* the fluid is more toxic to bacteria. This result is also obtained by placing the anode beneath the cathode; thus allowing the liberated chlorine to travel upwards towards the cathode. All these modifications, by facilitating the production of the sodium hypochlorite, increase the disinfecting value of the fluid.

For the disinfection of culture tubes, slides, etc., I would suggest the adoption of the following method: a glass jar (*i.e.* museum jar type) should be three-quarters filled with a 10–20% solution of common salt and an electric current passed through the fluid, using carbon electrodes.

The current for this purpose is best obtained from the ordinary lighting circuit and must be taken through a resistance of some kind, preferably the ordinary bench-light used for microscope illumination. For the experiments referred to here a 32 candle-power lamp was used, the jar was 10" high and 7" diameter, the carbon electrodes were 9" high, 5" wide and $\frac{1}{4}$ " thick.

In reality one of the cords of the ordinary lamp used for microscope illumination was cut, the copper wires at the severed points being bared for a distance of two inches. Each end of the wire was then tied separately to carbon plates (*c'* and *c''*) through a small hole previously bored into them. The exposed wire was thickly coated with varnish, paraffin wax, or sealing wax. It will thus be seen that, technically speaking, the electrodes of the disinfecting chamber were placed in series with a 32

candle-power carbon-filament lamp. Experiments made with the apparatus showed that opalescent emulsions of bacilli were rapidly disinfected. In the case of water sterilization could be readily accomplished by the addition of a minute percentage of sodium chloride, the other factors being so regulated that very shortly after electrolysis no alteration in taste or smell could be detected; the chemical tests for the presence of chlorine, etc. also yielding negative results.

Several experiments were made with spores of *B. anthracis*¹ and although the spores were undoubtedly very numerous, all attempts at cultivation after electrolysis showed the spores to have been destroyed.

To detail a further experiment, an old plate culture, thickly coated with *B. anthracis* and other sporing organisms, was placed in the apparatus. Within a few minutes the agar was colourless and films of

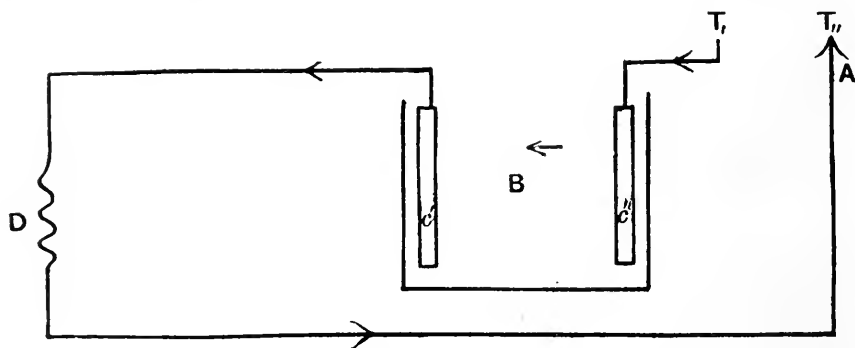
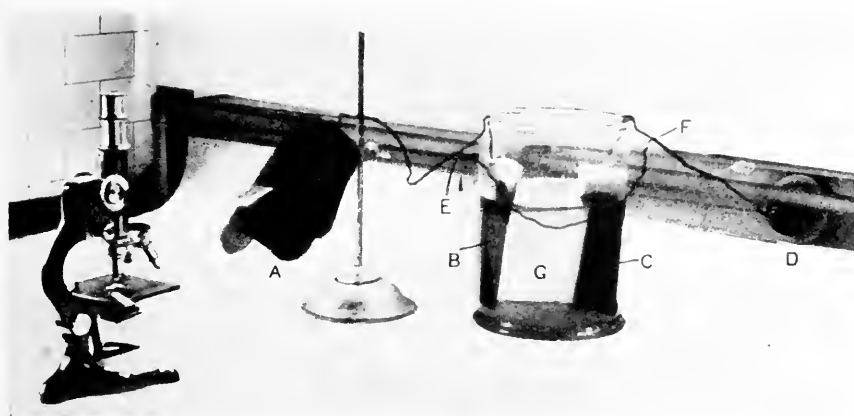


Fig. 1. Diagram showing the electrical connections where T' and T'' are terminals of an ordinary lighting circuit; B is a glass jar, c' and c'' are carbon electrodes; D is a resistance (electric globe). The arrows indicate the direction of the current.

the growth were floating in the fluid. The growth obtained by spreading a $\frac{1}{4}$ square inch of the floating film over the surface of an agar tube after the expiration of $\frac{3}{4}$ of an hour was very feeble, and at the end of $1\frac{1}{2}$ hours—the time at which the next culture was made—the floating films were sterile and most of the agar dissolved.

The lethal value of the fluid is increased by an increased candle-power lamp and also, of course, by the prolonged passage of the current, while, substantially speaking, so long as salt remains undissociated the fluid can be regenerated no matter what material, within reason, is placed in it for disinfection.

¹ Glynn, E. E. and Lewis, F. C. (1912). Detection of anthrax spores in industrial material. *Journ. Hygiene*, xii. 2, June, 1912, pp. 227-244.



The apparatus in use:

A, microscope lamp.

B, *C*, carbon electrodes.

E, *F*, bifurcation of wire.

G, jar of salt solution.

In conclusion I wish to draw attention to the advantages of this system of disinfection.

1. The bactericidal power of the solution obtained is maintained for several days even when the passage of the current is discontinued.

2. The bactericidal power of the fluid can be easily regenerated. The current need not be used except as and when required for laboratory purposes. The method, therefore, is economical.

3. While using electricity for microscope purposes useful disinfecting work is also being accomplished.

4. The apparatus is simple in arrangement and can be used on any bench where suitable current is available. The use of the apparatus materially assists the cleaning of culture tubes etc.

5. The apparatus can be used with confidence as to the efficiency of the disinfection.

In connection with this communication my best thanks are due to Professor J. M. Beattie for his criticisms and suggestions, and to Professor Ernest Glynn in whose laboratory the work was commenced.

A CONTRIBUTION TO THE STUDY OF THE
COMPLEMENT FIXATION REACTION IN
TUBERCULOUS ANIMALS.

BY LEONARD S. DUDGEON, F.R.C.P. LOND.

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(With 12 Charts.)

EXPERIENCE gained from a previous enquiry on this subject in human tuberculosis led to the belief that much information might be derived from an investigation of the complement fixation reaction in animals experimentally infected with tubercle bacilli. Accurate dosage can be measured and the true path of infection is definitely known. Various enquiries were suggested and investigated by the detailed examination of rabbits and guinea-pigs; the latter class of rodents were used in batches of six to twelve in number, as otherwise the individual differences between animals in the same group of experiments are entirely overlooked. My cultures of the human tubercle bacillus were obtained by inoculating guinea-pigs with the sputum¹ from typical cases of pulmonary tuberculosis at the Brompton Hospital Sanatorium at Frimley. Pure cultures of the bacillus were obtained from the infected guinea-pigs and cultivated on Dorset's egg medium, so that within a period varying from 14 to 21 days an abundant growth was obtained. The culture of the bovine bacillus was supplied to me by Professor Delépine who obtained it directly from the tissues of an infected cow, and subcultures were kept going on Dorset's egg medium. In every experiment without exception the animals were infected with definitely known quantities of the human or bovine bacillus. These were obtained by carefully scraping the growth off the surface of the egg medium and weighing it

¹ N.B. I am indebted to Dr W. O. Meek, Director of the Sanatorium, for supplies of the tuberculous sputum.

on sterile platinum foil, while in some cases (for comparison) a portion of the growth was dried in a desiccator before it was weighed. The untreated or dried bacilli were then shaken in a known quantity of sterile saline, so that a perfect emulsion free from clumps was obtained. The bacilli were kept in the dark in brown stoppered bottles and were always employed within a few days of their preparation.

The rabbits were weighed once a week during the observations and as far as possible animals of the same size and weight were employed in each group of experiments, but as regards guinea-pigs, only a rough method was adopted for their assortment.

The rabbits were always tested to ascertain whether the blood reaction was negative before inoculation, but it was only necessary to discard one animal out of the entire series. These animals were bled at least once a week during the period they were under observation, while in some cases it was necessary to bleed them much more frequently. The guinea-pigs were bled from the axillary artery at the end of the experiments. The serum was heated in the usual way and always tested within forty-eight hours, but comparisons were also made with "stored serum."

Observations on the blood in man can be made at definite periods in relation to certain clinical phenomena, or at rest, so as to avoid any question of the much discussed subject of auto-inoculation. In rodents, however, similar advantages cannot be seriously considered.

A complete post-mortem examination was carried out on every animal, and the various tissues were microscopically examined, as it is well recognised at the present day that naked eye observation alone can be disregarded.

Antigens.

Dudgeon, Meek, and Weir¹, in a preliminary communication on the complement fixation reaction in tuberculosis, described the preparation and the merits of certain antigens which they had employed; in their opinion the most satisfactory antigen contained the bodies of the tubercle bacillus. A vast amount of work on this subject has been completed since their paper was published with the result that this bacillary antigen has been practically abandoned. It is of undoubted value for determining a positive reaction but for titration purposes it cannot be regarded as ideal.

¹ Dudgeon, L. S., Meek, W. O., Weir, H. B. "A Preliminary Inquiry on the Value of the Complement Fixation Reaction in Tuberculosis." *Lancet*, Jan. 4th, 1913.

Experience has shown that this reaction is beset with difficulties, even greater in tuberculosis than in other conditions, but these can be largely overcome when a thoroughly satisfactory antigen is obtained. The first useful bacillary free antigen employed was made by extracting for long periods of time human tubercle bacilli cultivated on Dorset's egg medium from cases of pulmonary tuberculosis. These bacilli were weighed, then frozen and thawed over solid CO₂ and finally shaken with glass beads daily for some weeks, by which means a definitely specific saline antigen is procured, but if the bacilli are dried previously *in vacuo* and then extracted with distilled water instead of normal saline an antigen is obtained which possesses more active properties.

Extracts were also made with chloroform, benzene, acetone and ether, but finally a most satisfactory antigen was obtained, which is now used entirely for this reaction. It is prepared by a special extraction of living tubercle bacilli with absolute alcohol¹. It gives a beautiful mixture with normal saline, absolutely free from bacillary bodies and when carefully titrated leads to most satisfactory results, but a quantity of fresh and active tubercle bacilli is required for the preparation of this antigen and it is not fully active for some weeks. I have compared it with an unlimited number of others prepared by various methods and I consider it to be vastly superior to any other. No standard of activity can be given, as each supply must be judged on its own merits by most careful titration.

The Haemolytic Mixture.

A highly potent haemolytic immune-body of at least three times the strength of the minimum lethal dose was always employed, and a 5 per cent. suspension of thoroughly washed sheep red cells in normal saline.

Complement.

Fresh guinea-pig complement was diluted with normal saline on each occasion until the greatest dilution of the complement which acted efficiently was obtained.

It is absolutely essential for satisfactory work in the complement fixation test in tuberculosis to employ a powerful antigen, for preference a bacillary-free antigen, active fresh complement suitably diluted, thoroughly washed red cells and a powerful haemolytic immune-body,

¹ Deycke and Much (*München. med. Wochenschr.* 1913, No. 3) found that a neutral fat could be extracted from tubercle bacilli which could be employed with good results for complement fixation experiments on tuberculin and tubercle immunity.

while titration experiments should always be carried out with a tuberculous immune serum of known activity.

Each series of experiments were undertaken for the purpose of throwing some light on the question of immunity in tuberculosis and if possible, to trace a definite relationship between human and experimental tuberculosis.

It will be noticed by referring to the individual charts of the inoculated animals that doses with wide variations have been employed for these inoculation experiments, although the resulting lesions in the

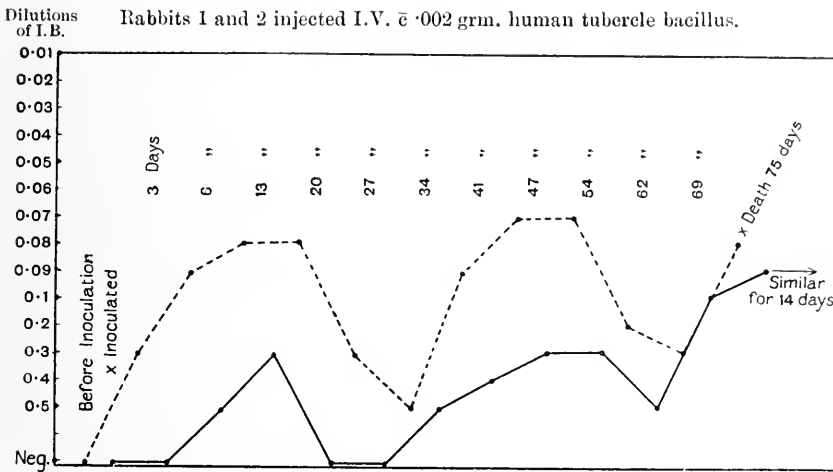


Chart 1.

Rabbit 1 represented by continuous line. Rabbit 2 represented by dotted line.

Weights:	Rabbit 1, before inoculation	2.610 grms.	Rabbit 2,	2.610 grms.
	on the 27th day	2.480 ,,	on the 34th day	2.050 ,,
	on the 69th day	2.190 ,,	on the 75th day	2.115 ,,

Result of post-mortem and microscopical examination.

Lungs: General caseous tuberculosis. Bacilli abundant in caseous areas.

Kidneys: Scattered areas of caseous tuberculosis with numerous bacilli present, but chiefly affecting pelvic portion.

Spleen: Rabbit 1. Very limited evidence of disease present.

rabbits appear to depend more upon individual characters of the host and the special properties of the individual bacillus isolated from a case of pulmonary tuberculosis in man than upon the actual doses employed.

We may perform as many control experiments as necessary, inoculate animals of similar size and weight by the same route, with the same

material, and with a similar dose, and, yet, the blood reaction may be widely different in each case, although the obvious clinical and pathological records are similar. Each rabbit requires special consideration, just as is necessary in the case of human beings, a fact which is strongly emphasised in the following experiment. Two rabbits were injected intravenously and two intraperitoneally with similar doses of the same emulsion of living human tubercle bacilli obtained from a case of pulmonary tuberculosis. The inoculations were made on the same dates and the blood was tested in an identical manner at the same intervals of time. Each rabbit received 1 c.c. of the emulsion which contained .002 gram. of bacilli suspended in normal saline.

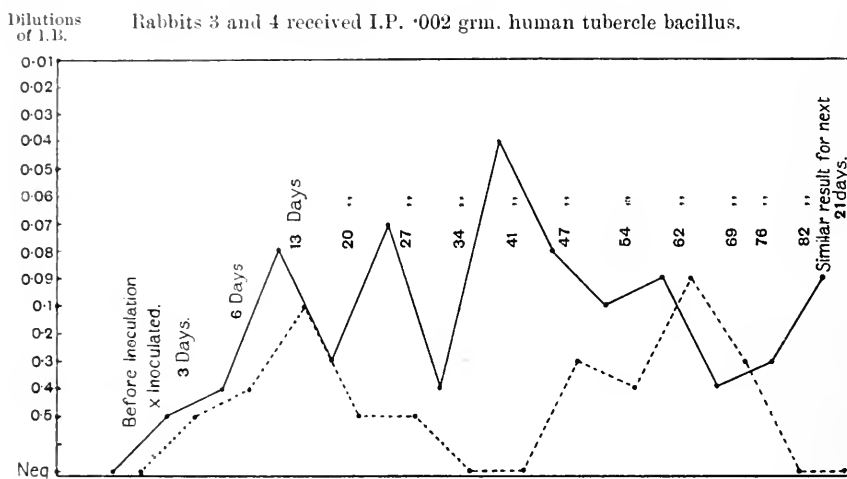


Chart 2.

Rabbit 3 represented by continuous line. Rabbit 4 represented by dotted line.

Weights :	Rabbit 3, before inoculation	2.960 grms.	Rabbit 4	2.530 grms.
	on the 34th day	2.750 "	41st day	2.600 "
	on the 96th day	2.290 "	82nd day	2.570 "

Result of post-mortem and microscopical examination.

Lungs: General caseous tuberculosis.

Peritoneum: Tubercles all through peritoneum.

When does the reaction first appear?

The earliest period at which the reaction was found to be positive occurred within four days from the time of inoculation. It is generally stated that 10 to 15 days are required for the development of the

allergic state from the time of the primary infection¹, previously little response to the infection has taken place, but then follows an inflammatory stage; fever develops, and local changes announce the fact that tubercle bacilli have produced a specific effect.

Three rabbits were inoculated intravenously with the same human bacillary emulsion and on the same date. One rabbit received 0.03, another 0.003, and the third animal 0.0015 gm. The rabbits which were inoculated with 0.03 and 0.003 gm. developed the reaction within a period of six days, while the rabbit injected with 0.0015 gm. did not react until after a period of fourteen days, but the final result in each case was similar. That the actual amount injected does not necessarily explain the difference, is shown by another experiment. Two rabbits of identical weight received the same dose intravenously, but there was a considerable difference between the periods when the reactions first developed. It is unnecessary to dwell further on this question, we will merely remark that there is abundance of evidence to show that the reaction period is not entirely dependent on dosage or virulence of the organism when injected intravenously or intraperitoneally.

Variations in the strength of the Reaction.

Attention has already been drawn to the technical details of the reaction, more especially to the fact that fresh guinea-pig complement suitably diluted was always employed, and that the same antigen was used week by week, while control observations were made with standardised immune-body so that every known factor in the titration experiments should remain constant during the whole course of the observations with various infected animals. The most satisfactory period in this work was reached when the antigens containing the bacillary bodies were replaced by the watery extract and finally by the most valuable antigen of all—the alcoholic. The most conclusive proof that constant results could be obtained by this method was demonstrated by titrating the sera each week, storing them, and repeating the whole series of observations at the end of the animal's life, six or eight weeks from the commencement of the experiment. It was then found that similar results were obtained, provided the sera were not contaminated.

¹ N.B. Allergy is a term used by Baldwin* and others to include all forms of "tuberculin" reaction, whether local or general, or any inflammatory process caused by tubercle bacilli or their products after infection has been established.

* Baldwin, Edward R. "Allergy and Re-Infection in Tuberculosis." *Johns Hopkins Hospital Bulletin*, July 1913.

It is, however, of considerable importance to realise that a strong positive reaction may be followed by a period when the reaction is absent, only to be succeeded by a reaction as strong as that which occurred before the negative period. Alterations in the character of the blood reaction observed at weekly intervals in this manner are of considerable importance, more especially in connection with the study of the blood in human pathology. It proves—if further proof is needed—that a negative reaction obtained as a result of a single blood examination may be totally disregarded. Two experiments will be cited to emphasise this point. A rabbit was inoculated intravenously with the human bacillus—a positive reaction developed in a few days and remained so for a period of one month, only to be succeeded by a negative period of 14 days duration, and then the reaction was again positive. A rabbit which had been injected intravenously with the bovine bacillus rapidly developed a powerful reaction which gradually declined, reaching the negative stage a month from the time of inoculation. This was followed by a short positive period just previous to the death of the animal. It only remains to point out that active immunisation of a tuberculous rabbit with a specific preparation may give rise to a negative reaction, as will be referred to later. In tuberculous animals, whether the disease is progressing slowly or rapidly, there is no appreciable alteration in their condition sufficient to explain these serological changes. It was thought that fluctuations in the weight of the infected animals might prove a clue to this particular point, but experience has proved that this is not so.

The Blood Reaction in Relation to Anaphylaxis.

The question which concerns us here is whether there is any relationship between the blood reaction and the hypersensitiveness of the infected animal. Sata, quoted by Austrian¹, claims to have shown a certain relationship between hypersensitiveness and the production of the anti-bodies. "Where hypersensitiveness develops, there immunity appears." Romer² found from a series of experiments upon animals that a primary infection was in itself protective against small re-infecting doses, but this immunity diminished as the second re-infecting dose was increased, finally reaching a state of hypersensitiveness to

¹ Austrian, C. E. "The Effect of Hypersensitiveness to a Tuberculo-Protein upon subsequent infection with *Bacillus tuberculosis*." *Johns Hopkins Hospital Bulletin*, Jan. 1913.

² Romer, P. K. (1908). *Beiträge z. Klinik d. Tuberkulose*, xi. p. 79.

large doses of tubercle bacilli. Romer considers that hypersensitivity to tubercle bacilli is quite distinct from hypersensitiveness to tuberculin.

Austrian's work on hypersensitiveness of the rabbit to tubercle bacilli was carried out by employing for the sensitising dose a tuberculous extract made by a modification of Baldwin's method; while the living bacillus isolated from human sputum was injected 21 days later. By this means rapidly fatal tuberculosis was set up such as occurs when the bovine bacillus is injected into rabbits. No observations, however, were made on the blood condition during this period of anaphylaxis.

Rabbits 1 and 2 inoculated I.P. \bar{c} 0.1 grm. of dried dead human tubercle bacilli and 21 days later with living human tubercle bacilli.

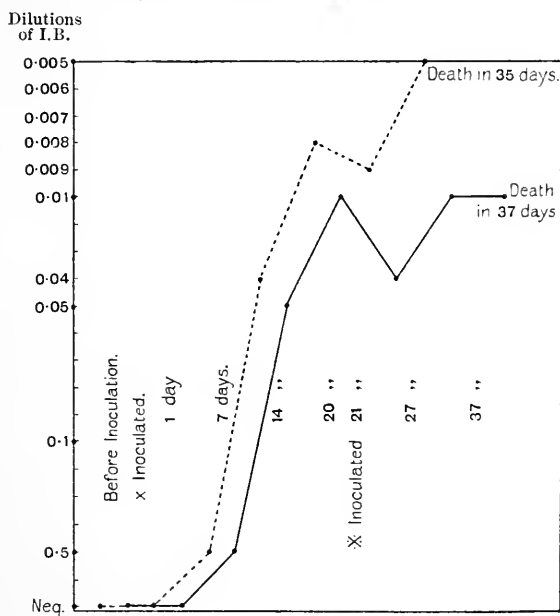


Chart 3.

Rabbit 1 represented by continuous line. Rabbit 2 represented by dotted line.

* Rabbit 1 received I.V. .0027 grm. living bacilli from human sputum, while Rabbit received I.V. .0013 grm.

Weights:	Rabbit 1, before inoculation	2.320 grms.	27 days later	2.100 grms.
	Rabbit 2, ,, ,,	2.560 ,,	27 ,,	2.290 ,,

Post-mortem and microscopical examination.

Fibro-caseous tuberculosis of the peritoneum.

General caseous tuberculosis of the various viscera.

Tubercle bacilli abundant in the caseous areas.

In my experiments rapidly fatal tuberculosis was produced by the infecting dose which was administered about 20 days after a sensitising intraperitoneal injection of dead tubercle bacilli. The main object was to incite a rapidly fatal disease by means of tubercle bacilli isolated from human tuberculous sputum, and to observe the blood reaction while the animals were suffering from rapid tuberculosis as compared with the period of disease incited by dead bacilli.

If we take as examples rabbits 1 and 2 of this series—each animal was of somewhat similar weight and each received 100 mgrms. of dead

Two Rabbits, X and Y, inoculated I.P. c 0.1 gm. dead human bacilli and 22 days later c living human bacilli.

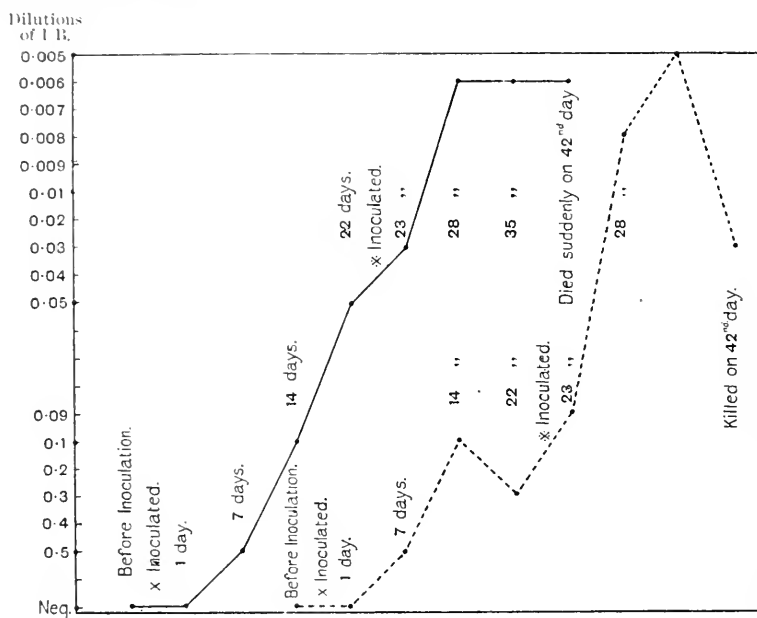


Chart 4.

Rabbit X is represented by the continuous line. Rabbit Y is represented by the dotted line.

* Rabbit X received 0.003 gm. living human bacilli, while Y received 0.0021 gm.

Weights:	Rabbit X, before inoculation	3.230 grms.	Rabbit Y	3.410 grms.
	37 days later	2.630 „	42 days later	3.000 „

Post-mortem and microscopical examination.

Abdomen: Very extensive fibro-caseous tuberculosis throughout peritoneal cavity. Great omentum greatly thickened. Tubercle bacilli very abundant. Tuberculosis extended down the cord on each side. Caseous tuberculosis of both testicles. Tubercle bacilli very abundant.

Liver, Spleen and Kidneys: Areas of caseous tuberculosis.

Lungs: Diffuse caseous tuberculosis. Tubercle bacilli very numerous.

tubercle bacilli. In each case a positive blood reaction developed which soon reached a high degree of activity. Twenty days after the first injection, each animal was inoculated intravenously with tubercle bacilli isolated from human sputum. One animal (R. 1) received 0·0027 grm., while the other (R. 2) was injected with 0·0013 grm. Both animals died a fortnight later from general tuberculosis, but during this period of fulminating tuberculosis, the blood reaction remained much the same as before the second dose was administered. Although this bacillus possessed active infective properties for the rabbit, as the control animal showed, yet, the sensitising dose had greatly increased the effect, while there was no reduction in the blood reaction in spite of the rapidly disseminated disease which was incited. The next three rabbits, to which full reference will be made, do not illustrate true anaphylaxis, but merely the effect of injecting a virulent bacillus into already sensitised animals. The control animal proved that the effect produced was not pure anaphylaxis. Rabbits *X* and *Y* received 100 mgrms. of dead human tubercle bacilli by the intraperitoneal route. A positive blood reaction occurred in each case within a week, and the strength of the reaction increased as time advanced. Twenty-two days later one animal (Rabbit *X*) and also a control rabbit, received 0·003 grm. of the bacillus isolated from human sputum, while rabbit *Y* was injected with a much smaller dose amounting to 0·002 grm. of the same bacillus. Although the second inoculation in each case was widely different, yet both animals developed general tuberculosis within a period of three weeks. While the active process was rapidly spreading, the blood reaction increased and reached a high degree of activity before death, and further it was found that each animal rapidly lost weight. The control rabbit had much less advanced tuberculosis than the rabbit *X*, which had been injected with a similar dose of the same strain of the living bacillus, and while rabbit *X* died of tuberculosis within three weeks of the second inoculation, the control animal was never *in extremis*.

The last rabbit (R. 22) which will be referred to serves to illustrate the effect of three intraperitoneal inoculations with dead human bacilli at intervals of about 17 days, while ten days after the last injection 0·006 grm. of living human bacilli was inoculated intravenously. The blood was examined each week and the effect carefully noticed. A highly active serum developed as a result of the inoculation with dead bacilli and still further increased after the intravenous injection of living bacilli, but became less marked in the final stages of the animal's

life. At the autopsy there was active and diffuse tuberculosis of the lungs, limited disease of the kidneys and spleen and chronic tuberculosis of the peritoneum.

True anaphylaxis set up in sensitised animals by the injection of a small dose of living bacillus occurred, but the blood reactions were similar to those recorded in the experiments referred to above. In conclusion these results emphasise that anaphylaxis and strong blood

Rabbit 22 inoculated on four separate occasions with human tubercle bacilli, (1) 0.04 gram. dead tubercle bacilli, (2) 16 days later 0.02 gram. of same emulsion, (3) 0.005 of same emulsion 35 days later, (4) 0.006 gram. of *living* tubercle bacilli I.V. 43 days later.

Dilutions
of I.B.

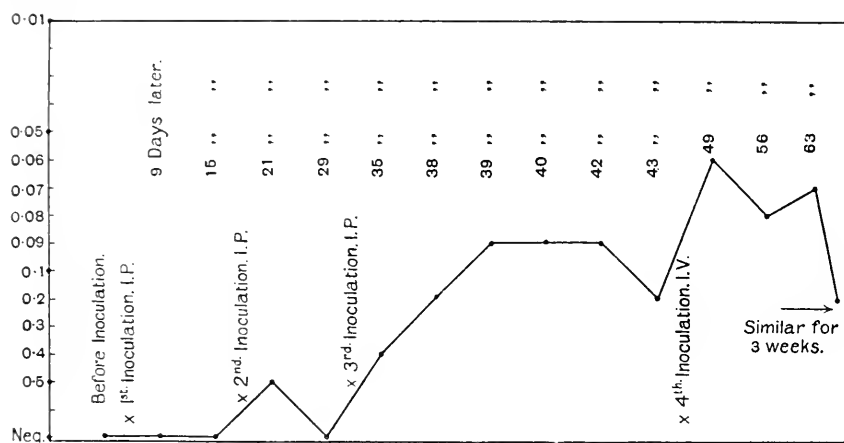


Chart 5.

Post-mortem and microscopical examination.

Lungs: Very oedematous. Diffuse tuberculosis. Caseation marked. Tubercle bacilli abundant.

Spleen: General tuberculosis.

Kidneys: Ditto.

Liver: Scattered tubercles present.

Peritoneum: Chronic fibrotic peritonitis.

reactions occur at one and the same time in the rabbit experimentally infected with tuberculosis—that there may be no alteration in the blood reaction in rabbits observed during the reaction period incited by the dead bacillus and the later stage of actively spreading tuberculosis.

Effect of Nuclein Injections on the Blood Reactions.

Various rabbits suffering from tuberculosis were injected subcutaneously with nuclein, the blood was collected at various intervals, and titration experiments were made with the serum. The total number of leucocytes and of polynuclear cells was estimated at the same periods as the blood examinations were made. It was not found, however, that nuclein caused any increase in the strength of the immune-serum, although the leucocytes and polynuclear cells showed the usual changes known to occur as the result of the injection of this substance. The blood was examined some days after the nuclein injections, but still no definite alteration had occurred which could be placed to the credit of the nuclein.

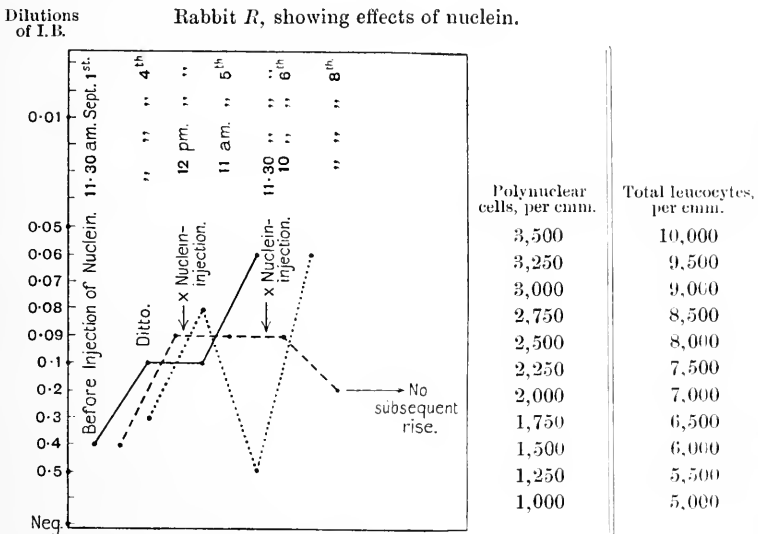


Chart 6.

- (1) Dotted line = Curve of polynuclear cells per cmm. (2) Interrupted line = Immune body content. (3) Continuous line = Curve of total leucocytes per cmm.

* Nuclein injections I.P. (1) 1 c.c., (2) 2 c.c.

One experiment (R. R) is cited in detail showing the effect on the leucocytes and blood serum as the result of the injection of nuclein intraperitoneally in a rabbit suffering from tuberculous peritonitis due to the injection of .04 grm. of dead human tubercle bacilli. As will be seen from the accompanying chart, no alteration was noted in the strength of the immune-serum, as measured by these methods of observation, although the usual leucocytic effects occurred.

*On the Results of the Injection of Tubercle Bacilli previously
"Treated" with Immune Tuberculous Serum.*

Four rabbits were injected intraperitoneally with living human tubercle bacilli which had been subjected to the following treatment. Human tubercle bacilli were cultivated on Dorset's egg medium from the spleen and liver of a guinea-pig which had been inoculated with the

Rabbits 1 and 2 were inoculated I.P. c 0.018 gm. of *treated* human tubercle bacilli.

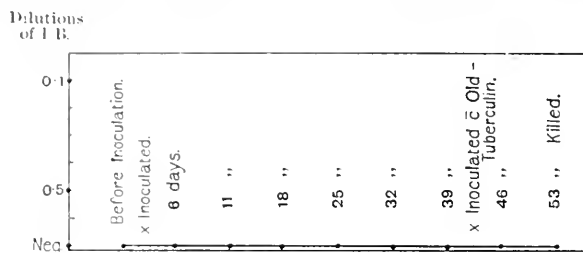


Chart 7.

Post-mortem and microscopical examination.

Nothing abnormal detected.

Rabbit 4 inoculated I.P. c 0.037 gm. of *treated* human tubercle bacilli and thirty days later I.V. with the same emulsion.

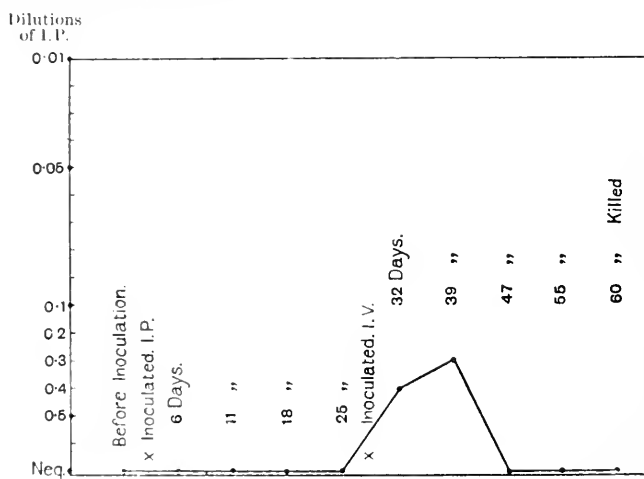


Chart 8.

Post-mortem and microscopical examination.

Lungs alone affected.

Scattered miliary tuberclosis of both lungs. Areas of consolidation are small and caseation is slight.

sputum from a case of pulmonary tuberculosis. An abundant growth was obtained in three weeks at 37°C ., and of this 0.185 gm. was mixed with sterile saline and shaken for one hour to break up the solid clumps. The saline was then pipetted off and the solid residue was thoroughly mixed in 2 c.c. of sterile saline, so that a very turbid suspension of bacilli was obtained. Two cubic centimetres of immune tuberculous rabbit serum was added and the total mixture incubated at 37°C . for 20 hours, then kept at room temperature for four hours. The bacilli were then thoroughly washed in sterile saline and finally suspended in that medium.

These results are of very great interest as we found that all four rabbits were free from active disease of the peritoneal cavity which was the seat of the primary inoculation, although the active pathogenicity of the bacillus¹ was proved by subcutaneous injection into guinea-pigs, as already referred to. Rabbits 1 and 2 received a large dose of the bacillus, but the blood examinations were negative without exception as can be readily seen from the accompanying charts. A month after the injection of living bacilli 10 mgrms. of Koch's old tuberculin were administered to both rabbits, but without any effect on the blood, and no alteration of the clinical condition in either case.

Rabbits 3 and 4 were injected on the first occasion with a much smaller dose of the bacillus than rabbits 1 and 2, but without any effect for a period of one month. Each animal was then inoculated with 0.0009 gm. of the same bacillary emulsion intravenously. In one instance (R. 4) a feeble blood reaction was obtained five days later, followed by a slightly stronger reaction, which, however, was succeeded by a negative period until the death of the animal. In the case of the other rabbit, the reaction was negative from first to last. Both rabbits were found to have early tuberculosis of the lungs at the post-mortem examination without any recognisable changes elsewhere, while rabbits 1 and 2 were apparently quite free from disease as already mentioned.

These results are of considerable interest as they were the only rabbits inoculated with treated bacilli. Three out of four failed to give a blood reaction at any period of examination while one did react for a very short period. Still further of importance is the fact that there was no tuberculous disease of the peritoneum which was the seat of injection, although both rabbits which had also been inoculated intravenously developed limited tuberculosis of the lungs.

¹ Both the untreated and the treated bacillus was found to be actively pathogenic to guinea-pigs.

The Results of the Examination of the Blood in Rapidly Fatal Tuberculosis in Rabbits.

The study of the blood in rapidly fatal tuberculosis in rabbits is a matter of comparative ease owing to the virulence of the bovine bacillus for this animal. In most cases of rapidly fatal tuberculosis in rabbits the reaction develops early and may be persistent during the short life of the animal, while little or no loss of weight may occur during the whole period of infection.

Rabbits 1 and 3 inoculated \bar{c} bovine tubercle bacilli. (1) R. 1, injected I.V. \bar{c} .0015 gram.
(2) R. 3, injected I.P. \bar{c} .0015 gram.

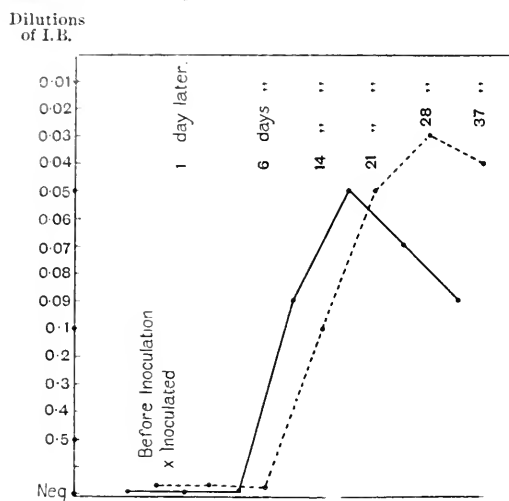


Chart 9.

Rabbit 1 represented by continuous line. Rabbit 3 represented by interrupted line.

Weights:	R. 1, before inoculation	2.050 grms.	R. 3, ...	2.850 grms.
	34 days later	1.920	37 days later	2.797

Rabbit 1. Result of post-mortem and microscopical examination.

Lungs: General tuberculosis. Both organs greyish white in colour and no obvious healthy tissue visible. Bacilli abundant in caseous areas.

General tuberculosis of Liver and both Kidneys.

Spleen: Tuberculous on microscopical examination.

Rabbit 3. Result of post-mortem and microscopical examination.

General tuberculosis of the Peritoneum, especially of the great omentum and extending along the cord.

Testicles: Caseous tuberculosis. Bacilli abundant.

Spleen: General tuberculous foci.

Liver: Scattered tubercles present.

Lungs: Early tuberculous change detected. No bacilli seen.

Three rabbits, 1, 2 and 3, were injected intravenously with 0.0015 and 0.0057 gm. respectively of the bovine bacillus, while the third animal received 0.0015 gm. intraperitoneally. The doses were large and the disease was rapid, with the result that death of all the animals occurred within a period of five weeks from the time of inoculation.

If we compare the bovine results with those obtained by the inoculation of a bacillus isolated from a case of pulmonary tuberculosis which was highly pathogenic to rabbits even in minute doses, we find that here also the reaction developed early.

The life of the animals inoculated with this bacillus was limited to a period of 3-4 weeks from the time of the inoculation, and in every instance miliary tuberculosis was recorded at the post-mortem examination.

The Effect of Tubercle Bacillary Emulsion and Tuberculin on the Blood Reactions and on the Progress of the Disease.

A certain number of animals infected with tuberculosis were subjected to inoculation with dead tubercle bacilli or their products, so as to determine as far as possible what effect was produced on the blood reactions. In some instances the dose employed for this purpose was determined by a comparison of the weight of man and rabbit, but in other experiments a very much larger dose was given than in cases of human tuberculosis.

A large number of experiments were completed on these lines and as useless repetition is unnecessary, a few examples will serve to illustrate the chief points which have been noted during these observations.

Two rabbits, *A* and *B*, had been inoculated intravenously with 0.03 and 0.0015 gm. of living human tubercle bacilli. A month later subcutaneous injections of tubercle bacillary emulsion were commenced and continued in increasing amounts at regular weekly intervals. It must be stated at the outset that the doses were distinctly large, but similar results were obtained with smaller doses. One rabbit lost weight, as will be seen by reference to the chart, the other also lost weight, but gradually recovered it. It was found at the post-mortem examination that both rabbits had advanced tuberculosis of the lungs and also tuberculosis of the kidneys. In neither case was the blood reaction increased by the injection, but on the contrary, as shown by the charts (*A* and *B*), a distinct reduction in the activity occurred. Numerous experiments have proved that as a rule tubercle bacillary

emulsion causes no increase in the blood reaction, while it not infrequently gives rise to a rapid fall or may be a completely negative result.

Still further no healing process or arrest in the course of the disease was detected in any animal whatever preparation was employed for the subcutaneous inoculations. Among those who administer tuberculin or allied preparations in cases of pulmonary tuberculosis in man some recognise its importance in dealing with the febrile state and in controlling the quantity of the sputum. In my opinion the *clinical*

Dilutions of I.B. Rabbit A inoculated I.V. \bar{c} 0015 gram. of living human tubercle bacilli.

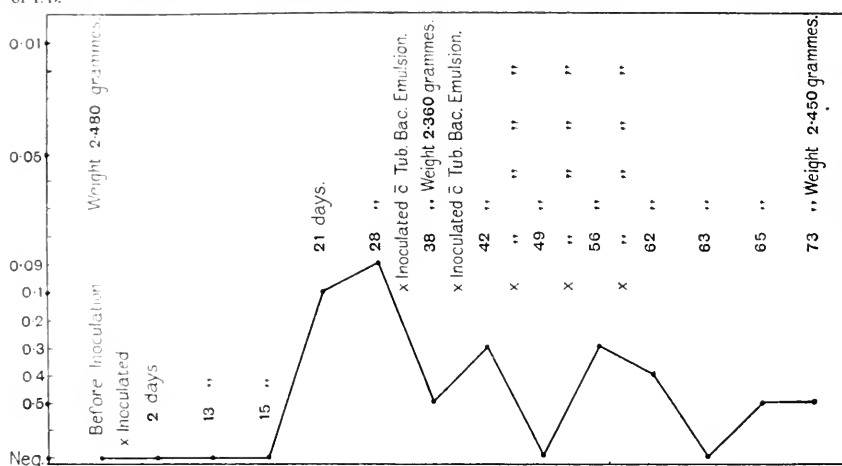


Chart 10.

Post-mortem examination.

Lungs: General tuberculosis, but posterior border more especially affected. Scattered tubercles all over both pleurae.

Kidneys: Tubercles seen on surface of left kidney.

Microscopy.

Lungs: Numerous scattered areas of caseation. Tubercle bacilli abundant.

Kidneys: Scattered tuberculous foci throughout both kidneys.

aspect of this disease in man and in infected rabbits has little in common and, therefore, it is unwise, because anti-tuberculous preparations are of little value in the treatment of tuberculosis in the lower animals, to condemn the use of specific preparations for the relief of certain symptoms of pulmonary tuberculosis in man. The charts of rabbits C and D show the effect of injecting two animals with dead

bovine bacilli intravenously and then treating the animals with subcutaneous doses of old tuberculin. Here we find a reduction in the immune body content of the serum as may occur as a result of this method of inoculation. The blood was frequently examined and the injections of old tuberculin were made at intervals of two and five days.

Both animals were killed three weeks from the time of the inoculation of the dead bacilli intravenously and the lungs showed a type of chronic pneumonia.

Rabbit *B* inoculated I.V. \bar{c} 0.03 gram. of living human tubercle bacilli.

Dilutions
of I.P.

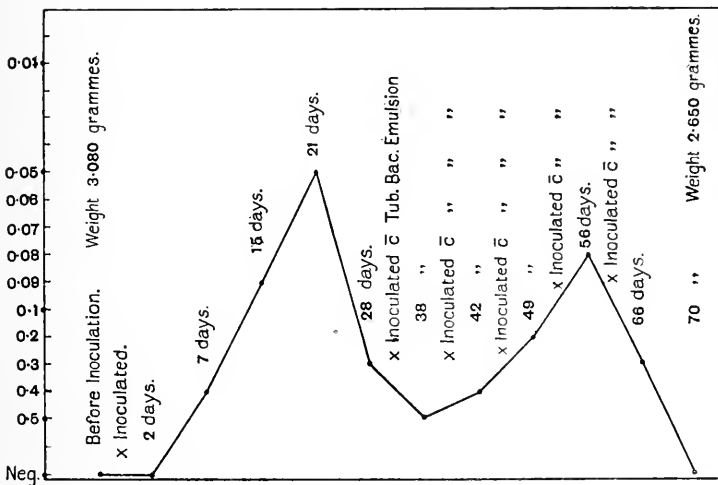


Chart 11.

Post-mortem and microscopical examination.

Similar results to Rabbit *A*, but disease more advanced, and *Spleen* shows general tuberculous foci.

Results obtained with Pleural and Peritoneal Exudates.

In certain instances effusions have been found at the post-mortem examinations on the infected animals. In nearly every instance the effusion has given a positive reaction, although the blood reaction may have been negative at the corresponding period, and also there was evidence to show that an effusion may be much more strongly positive than the blood serum. Dudgeon¹ pointed out in his Croonian Lectures

¹ Dudgeon, Leonard S., Croonian Lectures. "The Pathology of Immunity as concerns the Aggressins." *Lancet*, June and July, 1912.

that the exudate which collects in the peritoneum of immunised animals at the end of one hour may have a greater power to excite phagocytosis than the blood serum.

Rabbits C and D inoculated I.V. c '0025 gm. of dried dead bovine bacilli.

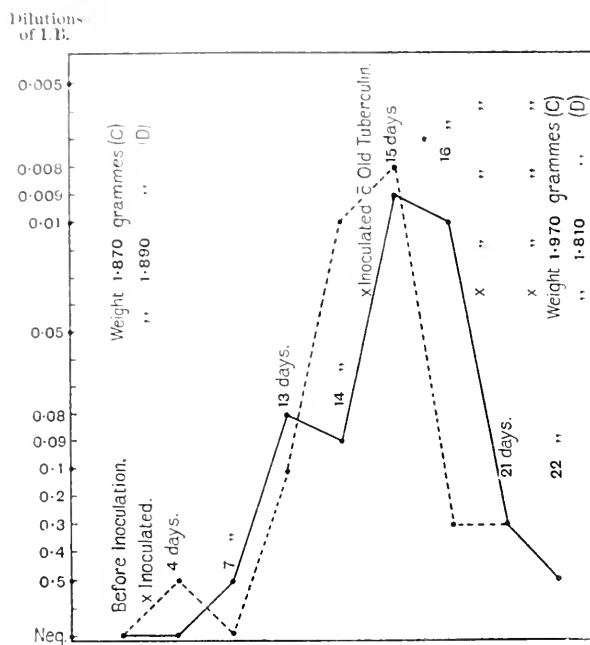


Chart 12.

Rabbit C represented by continuous line. Rabbit D represented by interrupted line.

Post-mortem and microscopical examination.

P.M. Nothing abnormal detected.

Microscopy: Chronic indurative pneumonia present. Alveolar walls very much thickened.

Patches of consolidations showing endothelial cells and giant cells present. Tubercle bacilli scarce.

An Examination of the Blood of Infected Guinea-Pigs.

These animals are not especially suitable for investigations of this kind, owing to the impossibility of obtaining sufficient blood for accurate observations at regular weekly intervals or may be even more frequently. For this reason single or duplicate experiments are quite valueless, but observations made on batches of these animals, so that individual anomalies can be recognised, afford the most satisfactory method.

Sixteen guinea-pigs were inoculated subcutaneously with 0.001 gm. of virulent human tubercle bacilli. Three were killed ten days later and all gave a positive reaction, while in most instances those killed at longer intervals when the disease was much more advanced, gave a negative reaction; although occasional guinea-pigs killed four or five weeks from the time of inoculation at a period when they were suffering from general tuberculosis not only gave a positive, but a strong reaction.

A batch of guinea-pigs were inoculated subcutaneously with 0.00275 gm. of dried living human tubercle bacilli. The animals were killed at varying intervals so as to determine when the reaction first appeared. It was generally found that this occurred about the tenth day of the disease, at a time when the obvious post-mortem appearances are mainly confined to the area of inoculation and to local glands. By no means all guinea-pigs give the reaction at this period, but some out of every batch will generally be found to react.

A series of guinea-pigs were inoculated intraperitoneally with human tubercle bacilli in doses of 0.002 or 0.001 gm. These animals were also killed at varying intervals to determine the periods at which the reaction first appears. In one case the peritoneal fluid gave a positive reaction seven days from the time of inoculation, although the blood of the animal failed to react. The period at which the blood reaction most commonly develops is about the tenth to the fourteenth day.

Animals inoculated with dead bacilli develop the reaction usually at a later period of infectivity than those infected with the living organism. It appears that quite a high percentage of guinea-pigs with very advanced disease, in fact, animals dying of general tuberculosis, may quite fail to give the blood reaction.

THE COMPLEMENT FIXATION REACTION IN TUBERCULOSIS.

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IN a previous paper (*Lancet*, 4. I. 1913), we published a preliminary report on this subject and are now able to give further particulars of our results and to discuss in detail certain points arising from them.

We have examined the blood from 234 cases of pulmonary tuberculosis (in all of which tubercle bacilli had been found in the sputum). Many of these were examined on several occasions.

Of these 234 cases, 209, or 89·3 %, gave a positive reaction; 194 on the first examination, while of the 15 which were negative on the first examination, 11 were positive on a second and 4 on a third.

In 25 cases a positive reaction was not obtained; of these 25, 11 were examined once only, 11 twice, 2 three times and 1 four times.

Out of 33 cases of "surgical" tuberculosis a positive reaction was obtained in 20 and a negative in 13.

A certain number of observations upon the sera of cases diagnosed clinically as tuberculous but without bacteriological proof and a few upon sera obtained from the post mortem room are disregarded as the numbers are too limited to allow any conclusion of value to be drawn.

The Technique. As our object has been largely to determine the quantity of immune-body present in different sera or in serum from the same case upon different occasions, careful standardisation of all

reagents has been necessary in order to render the "immune serum" the one unknown factor in the mixture.

The question of standardisation and of the antigens employed are dealt with in full by one of us (L. S. D.) in the preceding paper (this *Journal*, pp. 52-71).

To detect variations in the amount of immune-body present in the sera we have employed the method of diluting the sera with normal saline.

Any serum which gave a positive reaction when undiluted was further investigated in increasing dilutions until a point was reached at which no reaction was obtained.

The dilutions of serum commonly employed were 1 in 2, 1 in 10, 1 in 20, 1 in 100 and if necessary 1 in 200 and 1 in 1000, etc.

In a few of the cases a full series of intermediate dilutions was employed with results of considerable interest, as instanced by the results obtained with the sera of cases to whom nuclein had been given. (See Table.)

The adoption of this procedure in every instance would have necessitated the investigation of a much smaller number of cases and our primary object has been the detection of marked changes in the strength of the reaction.

In about half of the cases investigated a parallel series of tests were made using various dilutions of the antigen. As we met with no instance in which a serum or dilution of serum gave a positive reaction with dilute antigen after failing to do so with antigen of full strength, this procedure was abandoned.

In quite a considerable number of cases a positive reaction was obtained with a serum dilution of 1 in 100, while in a few instances the reaction was still positive with a serum dilution of 1 in 1000. No human serum we have yet examined has given a positive result in a greater dilution than this.

While in many cases the amount of immune-body as estimated by titration remains remarkably constant over long periods, in others, striking variations occur. For instance, a serum which, on a certain date, gave a positive reaction in a dilution of 1 in 1000, was found a fortnight later to give a negative reaction in a dilution of 1 in 2. Another serum which on one day only gave a positive reaction when undiluted, was found, on the following day, to give a reaction in a dilution of 1 in 10 while three days later it was again positive only when undiluted.

Since in all the cases of pulmonary tuberculosis considered in this report, tubercle bacilli were present in the sputum, we are not in a position to speak with authority on the value of the reaction in the diagnosis of this condition. To be of value in this respect the reaction must be shown to be present in individual cases before bacteriological proof is available.

It is quite certain that the absence of a reaction on one occasion is of little value in diagnosis for, as is shown by some of the examples quoted in the second portion of this paper, the serum of undoubted cases of phthisis may fail to give a reaction for protracted periods.

We have met with no positive reaction among our control sera obtained from presumably healthy individuals of the middle class.

The effect of nuclein solution upon the reaction.

In several cases a solution of nuclein was administered by mouth or hypodermically to phthisical persons with the object of noting any subsequent change in the amount of immune-body demonstrable in the blood serum.

In no case could any variation be detected.

The observations on two of these cases are shown in the foregoing table.

In two cases where demonstrable "immune-body" had disappeared from the blood serum after previously being present, the administration of nuclein solution brought about no return of the "immune-body."

The effect of therapeutic doses of tubercle bacillary emulsion upon the reaction.

Since our preliminary report was written we have had opportunity of examining sera from a considerable number of phthisical people who were receiving continued small therapeutic doses of bacillary emulsion ($\frac{1}{500,000}$ to $\frac{1}{300}$ mgm.). The variations in the strength of the reactions observed with the sera of these patients have not convinced us that small doses of this emulsion have any constant or marked effect upon the amount of immune-body in the serum.

In several instances marked improvement in the condition of the patient with diminution in the amount of immune-body or its complete disappearance from the serum has coincided with or followed gradually increasing doses of the drug.

OBSERVATIONS ON THE IMMUNE-BODY CONTENT OF THE BLOOD SERUM IN PULMONARY TUBERCULOSIS, AS DETERMINED BY MEANS OF THE COMPLEMENT FIXATION REACTION.

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*(From the Department of Pathology of St Thomas's Hospital
and The Brompton Hospital Sanatorium.)*

THE object of this portion of the investigation was to determine

(a) Whether the amount of immune-body present in the sera of cases of pulmonary tuberculosis, bore any constant relation to the stage of the disease and the condition of the patient.

(b) Whether well-marked clinical phenomena, *e.g.* periods of fever, attacks of pleurisy etc., were associated in any definite manner with variations in the amount of immune-body in the serum.

(c) Whether gradual definite and sustained improvement or deterioration in a patient's condition was accompanied by increase or decrease of the specific immune-body in the serum.

The method adopted was repeated examination of the sera of a large number of phthisical people at more or less frequent intervals, extending over a period of some months, with special reference to marked changes in the patient's condition.

In all cases tubercle bacilli were found in the sputum on one or more occasions while the persons were under observation.

A. The relation between the stage of the disease and condition of the patient and the amount of immune-body in the serum.

In this connection only those cases are considered which could easily be included in one of the three following classes :

(1) Cases with physical signs of extensive pulmonary disease, copious sputum, impairment of nutrition and general health, seriously impaired working capacity and an apparently bad prognosis.

(2) Cases of limited disease, not of long standing, without, during the period of observation, serious constitutional symptoms and whose capacity for work was not greatly affected.

(3) Cases of long standing disease, with physical signs of greater or less extent, without serious constitutional symptoms and in whom a diagnosis of "chronic fibroid phthisis" might be fairly made.

For purposes of comparison it is necessary to fix an arbitrary standard. This has been done by saying that any serum which gave a positive reaction in a 1 in 10 dilution, gave a "strong" reaction.

Adopting this standard, out of 102 sera from cases in class (1) ("severe" cases), 65 gave on one or more occasions a "strong" reaction while 37 gave always a weaker reaction or no reaction.

Of 43 sera from cases in class (2) ("slight" cases), 13 gave on one or more occasions a "strong" reaction and 30 gave always a weaker reaction or no reaction.

Of 60 sera from cases in class (3) ("chronic fibroid cases"), 35 gave on one or more occasions a "strong" reaction and 25 gave always a weaker reaction or no reaction.

These figures are vitiated by the fact that the sera from all cases were not examined with equal frequency; in some cases the serum was examined only on two or three occasions, in others on as many as fifteen occasions. Had this source of error been avoided and each serum examined fifteen times, it is probable that a "strong" reaction would have been met with in a somewhat greater proportion of the sera.

It would appear that the greatest amount of immune-body is likely to be found in sera obtained from severe cases or those with extensive lesions, and, as a matter of experience, this has proved to be the case.

It is, however, quite impossible to foretell the presence or absence of demonstrable immune-body or the amount of such immune-body in the serum of any particular person on general or clinical grounds. Two sera from two comparable cases may show widely different properties.

For example, the following two pairs of cases, while very similar from a clinical point of view, gave totally different results on examination of the serum.

In all the following examples abbreviations have been employed for convenience. C. F. R. +, C. F. R. -, mean, respectively, "complement fixation reaction present" and "complement fixation reaction absent."

The strength of the reaction is expressed quantitatively by the terms, "serum dilution '5," ".01," etc., which mean that the highest dilution of the immune serum with which the reaction was obtained was a 1 in 2, 1 in 100, dilution etc. The term "full-strength serum" means that the reaction was obtained with undiluted serum but not with a 1 in 2 dilution. (In a very few of the later observations, where only the special alcoholic antigen was employed, the reaction was always performed with diluted serum.)

As stated above, tubercle bacilli were present in the sputum of all the following examples:

A. Two "chronic fibroid" cases.

1. Male aet. 42, 20 years' history. Signs of fibrosis of lung (not extensive). General condition good. Working capacity not seriously impaired. Scanty sputum. Temperature consistently subnormal.

Oct. 11.	C.F.R. +.	Serum dilution '1.
Nov. 1.	C.F.R. +.	Serum dilution '1.
Nov. 25.	C.F.R. +.	Serum dilution '05.
Dec. 16.	C.F.R. +.	Serum dilution '05.
Jan. 1.	C.F.R. +.	Serum dilution '01.
Jan. 28.	C.F.R. +.	Serum dilution '05.
Feb. 11.	C.F.R. +.	Serum dilution '05.

2. Male aet. 23, 8 years' history. Fibrosis of upper lobes. Scanty sputum. General condition good. Working capacity not impaired. Temperature consistently subnormal.

Nov. 8.	C.F.R. -.
Nov. 26.	C.F.R. -.
Dec. 18.	C.F.R. -.
Jan. 21.	C.F.R. -.

B. Two moribund cases.

3. Male. Very extensive disease. Great emaciation. Bedridden for some months before death. No serious complications.

Nov. 8.	C.F.R. +.	Serum dilution '1.
Nov. 13.	C.F.R. +.	Serum dilution '1.
Nov. 21.	C.F.R. +.	Serum dilution '5.
Dec. 3.	C.F.R. +.	Serum dilution '5.
Dec. 16.	C.F.R. +.	Serum dilution '01.
Jan. 6.	C.F.R. +.	Serum dilution '5.
Jan. 14.	C.F.R. +.	Serum dilution '01.
Feb. 5.	C.F.R. +.	Serum dilution '5.
Feb. 19.	C.F.R. +.	Serum dilution '01.
Mar. 5.	C.F.R. +.	Serum dilution '01.
Mar. 28.	Death.	

4. Female. Uncomplicated pulmonary tuberculosis.

May 26.	C.F.R. -.
June 9.	C.F.R. -.
June 26.	Death.

B. The relationship between variations in the amount of immune-body in the serum in pulmonary tuberculosis and exacerbations of the disease, *e.g.* periods of fever, acute pleurisy, extending lesions, etc.

Repeated examinations of the sera from a number of cases over extended periods show widely varying results. Periods of fever, with or without definite signs of extension of the tubercular process in the lungs or attacks of acute pleurisy, may occur without any marked change in the amount of immune-body, or they may be accompanied by a considerable increase or decrease of this substance.

On the other hand, profound changes in the strength of the reaction occur from time to time in tuberculous subjects without any accompanying change in the clinical condition.

This being so, it seems advisable to cite some of the more interesting examples met with without further comment.

5. Reaction appearing shortly after an attack of acute pleurisy.

Male. Extensive disease. Serious constitutional and pulmonary symptoms. Afebrile.

On May 29th, acute pleurisy with effusion, with fever which lasted till June 11th.	May 19.	C.F.R. - .	
	June 6.	C.F.R. - .	
	June 11.	C.F.R. - .	
Between June 11th and 15th the effusion was rapidly absorbed. The temperature fell below 98·4 on June 11th and remained subnormal subsequently.	June 18.	C.F.R. + .	Serum dilution ·5.
	June 25.	C.F.R. + .	Serum dilution ·5.
	July 14.	C.F.R. - .	
The patient slowly convalesced from the attack of pleurisy but the original severe symptoms persisted.	Aug. 14.	C.F.R. - .	
	Aug. 28.	C.F.R. - .	
	Sept. 18.	C.F.R. - .	

6. Variations in the reaction during an attack of acute pleurisy.

Male. Extensive disease. Severe constitutional and pulmonary symptoms. Afebrile.

	Feb. 19.	C.F.R. + .	Full strength serum.
May 5th. Acute pleurisy with effusion. Fever with marked oscillations of temperature. Gradual defervescence to a normal temperature on June 4th.	May 14.	T.° 101·8 C.F.R. + .	Serum dilution ·1.
	May 20. A.M.	T.° 98·6 C.F.R. + .	Full strength serum.
	May 20. P.M.	T.° 101·6 C.F.R. + .	Full strength serum.
	May 21. A.M.	T.° 99·2 C.F.R. + .	Full strength serum.
	May 21. P.M.	T.° 100·8 C.F.R. - .	
	May 29.	T.° 99·4 C.F.R. + .	Full strength serum.
From June 5th the temperature remained subnormal, the effusion was slowly absorbed and the patient very slowly regained his former condition.	June 5.	C.F.R. - .	
	June 11.	C.F.R. + .	Serum dilution ·5.
	June 18.	C.F.R. + .	Full strength serum.
	July 14.	C.F.R. - .	
	Aug. 28.	C.F.R. - .	

7. Persistent increase in strength of the reaction following acute pleurisy with effusion.

Male. Extensive disease. Severe constitutional symptoms. Much sputum. Afebrile.

	Jan. 20.	C.F.R. +.	Serum dilution .5.
Jan. 24th. Temperature rose to 103. Pleurisy developed followed by effusion. Temperature gradually fell and reached normal on Feb. 10th.	Feb. 5.	C.F.R. +.	Serum dilution .5.
Feb. 10th onwards. Afebrile. Slow convalescence. The effusion was slowly absorbed.	Feb. 19.	C.F.R. +.	Serum dilution .01.
	Mar. 5.	C.F.R. +.	Serum dilution .1.
	April 22.	C.F.R. +.	Serum dilution .05.
	June 11.	C.F.R. +.	Serum dilution .1.
	Sept. 4.	C.F.R. +.	Serum dilution .1.

8. Rapid change in the reaction during a febrile period due to acute pleurisy.

Male. Extensive disease. Ill. Much sputum. Afebrile.

March 26th. Acute pleurisy. The temperature rose to 102. No signs of effusion.	March 31.	C.F.R. +.	Full strength serum.
	April 1.	C.F.R. +.	Serum dilution .1.
	April 5.	C.F.R. +.	Full strength serum.
The temperature fell to normal on April 8th and remained so.	April 11.	C.F.R. +.	Full strength serum.

9. Persistent disappearance of the reaction following haemoptysis and extension of the disease.

Male. Old standing unilateral disease. Ill. Much sputum. Afebrile.

	Nov. 13.	C.F.R. +.	Full strength serum.
Nov. 24. Severe haemoptysis with fever. Temperature 102. Severe haemoptysis repeated almost daily for 8 days. The temperature fell on Dec. 3rd and remained subnormal. Patient very ill.	Dec. 12.	C.F.R. -.	
	Dec. 17.	C.F.R. -.	
On Dec. 21st the temperature rose to 103.8 and remained elevated for 10 days. Physical signs of extensive dissemination of the pulmonary disease.	Dec. 30.	C.F.R. -.	
The temperature became normal again on Jan. 3rd and remained so. The patient very slowly rallied and improved to a considerable extent.	Jan. 7.	C.F.R. -.	
	Jan. 22.	C.F.R. -.	
	Feb. 5.	C.F.R. -.	
	Feb. 19.	C.F.R. -.	
	Apl. 29.	C.F.R. -.	

10. Febrile attacks with little or no variation in the strength of the reaction.

Male. Limited disease. General condition good. Slight cough, and sputum. Afebrile.

	Feb. 27.	C.F.R. +.	Full strength serum.
March—April. Progress uneventful.			

April 30th. The temperature rose suddenly to 102 with expectoration of much caseous matter containing many tubercle bacilli.	April 30. T.° 102·0 C.F.R. +. Serum dilution ·5.
May 5th—June 11th. Afebrile. Progress uneventful. Little sputum.	May 1. T.° 101·0 C.F.R. +. Full strength serum.
	May 3. T.° 99·6 C.F.R. +. Serum dilution ·5.
June 11th. The temperature rose to 100. Sputum again abundant.	May 5. T.° 98·0 C.F.R. +. Serum dilution ·5.
July 16th. Afebrile. Progress since June 15th uneventful.	May 8. C.F.R. +. Serum dilution ·5.
	May 20. C.F.R. +. Full strength serum.
	June 12. C.F.R. +. Serum dilution ·5.
	June 18. C.F.R. +. Serum dilution ·5.

11. Reaction persisting until shortly before death.

Female. Uncomplicated case, 9 months' pyrexia, Jan. to Oct.

Oct. 18. T.° 101·8 C.F.R. +. Serum dilution ·1.
Oct. 26. T.° 100·0 C.F.R. +. Serum dilution ·5.
Oct. 30. T.° 100·0 C.F.R. +. Serum dilution ·5.
Nov. 13. T.° 100·8 C.F.R. +. Serum dilution ·5.
Nov. 25. T.° 100·6 C.F.R. +. Serum dilution ·5.
Dec. 2. Death.

12. Reaction becoming less marked before death.

Male. Pulmonary tuberculosis and tuberculosis of the intestines.]

May 30. C.F.R. +. Serum dilution ·1.
June 10. C.F.R. +. Full strength serum.
June 22. Death.
June 23. Serum obtained post mortem. C.F.R. - .

13. Variations in the reaction in an uncomplicated case going steadily downhill.

Female. Prolonged pyrexia.

Oct. 18. Slight pyrexia. C.F.R. +. Serum dilution ·1.
Oct. 25. „ „ C.F.R. +. Serum dilution ·5.
Oct. 30. „ „ C.F.R. +. Serum dilution ·5.
Nov. 8. „ „ C.F.R. +. Serum dilution ·1.
Nov. 25. „ „ C.F.R. +. Serum dilution ·5.
Dec. 12. Marked „ C.F.R. +. Serum dilution ·05.
Dec. 30. „ „ C.F.R. +. Serum dilution ·1.
Death a few weeks later.

14. Numerous observations in an advanced case going steadily downhill.

Male. Course mainly afebrile. Occasional slight pyrexia.

Oct. 4. Slight pyrexia. C.F.R. +. Serum dilution ·1.
Nov. 1. „ „ C.F.R. +. Serum dilution ·5.
Nov. 25. Afebrile. C.F.R. +. Serum dilution ·1.
Dec. 10. „ C.F.R. +. Serum dilution ·05.
Jan. 6. „ C.F.R. +. Serum dilution ·1.
Jan. 28. „ C.F.R. +. Serum dilution ·05.
Feb. 11. „ C.F.R. +. Serum dilution ·5.
Mar. 3. Slight pyrexia. C.F.R. +. Serum dilution ·1.
April 22. Afebrile. C.F.R. +. Serum dilution ·5.
May 20. „ C.F.R. +. Full strength serum.
June 11. „ C.F.R. +. Serum dilution ·5.

July	1.	Afebrile.	C.F.R. +.	Serum dilution '5.
July	2.	..	C.F.R. +.	Serum dilution '5.
July	3.	..	C.F.R. +.	Serum dilution '5.

Marked variations in the reaction without any corresponding evident change in the condition or health of the patient.

15. Male. Chronic fibroid disease of one apex. Scanty sputum. General condition excellent. Temperature subnormal.

Dec.	15.	C.F.R. +.	Serum dilution '01.
Mar.	3.	C.F.R. - .	
Mar.	12.	C.F.R. +.	Serum dilution '1.
Mar.	25.	C.F.R. +.	Serum dilution '5.

16. Male. Fibroid apical disease. General condition excellent. Scanty sputum. Temperature subnormal.

Nov.	1.	C.F.R. +.	Serum dilution '5.
Nov.	18.	C.F.R. +.	Serum dilution '5.
Jan.	21.	C.F.R. +.	Full strength serum.
Mar.	11.	C.F.R. +.	Serum dilution '1.

C. The relationship between sustained improvement or deterioration in the condition of phthisical patients and the amount of immune-body in their sera.

(a) Patients who had improved.

Only those cases are included which showed a complete loss of all symptoms (including loss of all sputum for some weeks), complete restoration of the general health and restored working capacity together with improvement in the physical signs in the lungs or, at least, no increase in the extent of such signs.

Serum was obtained from the patients on two occasions, when first seen and upon their discharge from a sanatorium. Observations made in the interval are disregarded.

Of 12 such cases, the reaction, as finally observed (compared with the reaction at the first examination), was stronger in 4, weaker in 3, and unaltered in 5.

(b) Patients whose condition was becoming worse.

Serum was obtained from a number of uncomplicated cases who were going steadily downhill, on two occasions, at an interval of some months. In some instances the subject was dying at the time of the second observation.

Of 20 such cases, the reaction at the time of the final observation was stronger in 7, weaker in 7, and unaltered in 6.

My thanks are due to Mr G. W. Smith for invaluable and ever-ready assistance.

THE RELATION BETWEEN THE CANCER AND DIABETES DEATH-RATES.

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Introduction.

EARLY in 1912 Professor E. C. C. Baly, F.R.S., acting on behalf of Mr Jessup, brought one of us a collection of Swiss statistics and requested that it should be analysed, with the object of measuring the relation between the death-rates of cancer and diabetes. In the course of this work we have been led to consider many problems of theoretical and practical interest, bearing partly on the method of analysis and partly on the results yielded; it would not be possible to deal with all these in a single paper and the present memoir is devoted to a study of the problem first suggested, viz. the relation between the cancer and diabetes death-rates. Since, however, this may be the first paper of a series, it will be convenient to deal at some length with the nature of the investigation and the motives which led us to undertake it.

In 1909 Dr G. D. Maynard published under the title of "A Statistical Study of Cancer Death Rates" (*Biom.* 1909-10, VII. pp. 278-304) an important contribution to the subject now under discussion.

In the first place Maynard set himself to determine whether the incidence of cancer varied with meteorological conditions. His material consisted of cities in the United States of America, each having over 100,000 inhabitants and he correlated the corrected cancer rate with sunshine in hours, mean temperature, rainfall and other meteorological conditions. In no case was a definitely significant result obtained.

It then occurred to him to correlate the cancer rate with that of

some other diseases and he was particularly led to consider diabetes in this connection, because (1) both diseases have very much the same age distributions, (2) both are on the increase, (3) the aetiology of both diseases is obscure.

The method he adopted was twofold. In the first place he correlated the absolute number of deaths from cancer (*c*) with the absolute number of deaths from diabetes (*d*), then correlated each with the absolute population (*p*) and finally worked out the partial correlation of cancer deaths with diabetes deaths for population "constant," *i.e.* r'_{cd} . In the second place the absolute numbers of deaths from each disease were corrected for age distribution by the use of the well-known correlation factors (for description see supplement to 65th annual report of the Registrar General) and the previous process repeated with these corrected totals.

These methods were employed in the case of 15 states and 40 cities. The results were that in every case a large and significant positive correlation was found. Thus, using uncorrected figures, the values were, $6896 \pm .0559$, $.9088 \pm .0303$ for cities and states respectively; and using corrected figures, $7325 \pm .0494$ and $.8258 \pm .0554$.

These very high values either (1) might indicate a close relationship between the two diseases in consequence of some common physiological factor; (2) might be due to some error of method or material; or (3) might be dependent upon some indirect cause of association, *e.g.* the influence of common occupation.

The first possibility which suggested itself was that some element of so-called spurious correlation had been introduced by the process of correcting for age distribution, which involved the application of certain common factors to both of the variables. This possibility has been made the object of a special inquiry by Pearson and Lee (*J. Roy. Stat. Soc.* 1910, LXXIII. 534) in which an elaborate method of neutralising this source of error is described and illustrated. The correction was applied to Maynard's data and it was found that no appreciable change was made in the value of the correlation. Pearson accordingly concluded that Maynard's results could not be accounted for in this way. We have ourselves further considered this point and have applied several tests to Maynard's results. Our work entirely confirms the view of Pearson and we have no doubt that whatever may be the cause of Maynard's results, methodological errors cannot have been significant sources of correlation. The nature of the data is a much more disputable subject.

The first question is as to whether the returns might not be untrustworthy and not real measures of the prevalence of the two diseases. Maynard investigated this point in the following way. In the first place he thought the registration of deaths might be very imperfect. This had been the subject of a special inquiry by the United States Census department in 1900. Enumerated deaths were compared with registered deaths and the result showed that, so far as the cities investigated by Maynard are concerned, the error never exceeded 9.5%; in only one city was the error greater than 6.5%, while the mean was 3.5%, with a coefficient of variation of 2.225. So that it would appear that the numbers of deaths which escape registration are not large enough to be a serious source of error. This does not of course enable us to form an opinion as to the accuracy of the diagnoses recorded on the certificates of death. As a check on this Maynard correlated the cancer and diabetes rates with deaths from ill-defined or unknown causes and found that the correlation was very small in both cases. In reply to the objection that in towns well equipped with competent medical men, the two diseases would figure less in the death returns because of operative treatment in the case of cancer and dietetic treatment in the case of diabetes and that the converse would hold in towns where medical skill was inferior or the population too ignorant to follow advice, Maynard urges that these cities do not probably differ greatly in such respects and that in neither disease is a cure effected, even by the best treatment, except in rare instances.

The most obvious cause of indirect association, other than those already mentioned, would be that certain occupations predispose to the development of both diseases. To test this Maynard correlated occupations with diabetes and with cancer rates. He found that the coefficient differed not only absolutely, but also in sign, *i.e.* that the occupations with a high cancer rate tended to exhibit a low diabetes and conversely. It did not therefore seem possible to explain the results in this way.

Having apparently exhausted the indirect or erroneous causes of correlation, Maynard offered a provisional explanation of his results. He writes: "Only one cause, it seems to me, will fit the facts as we know them, *viz.* the pressure of modern civilization and the strain of modern competition or some factor closely associated with these."

He suggests that the increasing tension of life owing to competition and nervous strain might account for the facts and points out that there is a high correlation between either rate and the prevalence of insanity

in the states used and that there is a similar correlation with the number of suicides in both states and cities.

Another point is whether the correlation might not be due to both rates increasing with the general unhealthiness of the district. This does not seem to be the case, because (1) the correlation between cancer and nephritis is very much smaller than between cancer and diabetes, and (2) Pearson found from Maynard's data that the correlation between cancer and deaths from all causes other than diabetes and cancer was markedly less than that between cancer and diabetes.

As will be seen, Maynard was thoroughly on his guard against material fallacies and both he himself and also Pearson have carefully and, we think, successfully guarded against the risk of error due to improper analytical treatment; the one point which we think it advisable to subject to further scrutiny is the question of diagnostic accuracy. The authorities of the Imperial Cancer Research Fund have referred to the curious difference between the recorded death-rates from cancer in Ireland and in the rest of the United Kingdom (*Scientific Reports on the Investigations of the I.C.R.F.* No. 2, Part 1; *The Statistical Investigation of Cancer*, by Drs Bashford and Murray, pp. 40-3). They point out that the death-rate from cancer in Ireland is very much smaller than in the rest of the United Kingdom. It is then noted that autopsies are performed in the Irish hospitals much less frequently than in England. Since many cases would not be diagnosed but for the post-mortem findings, it follows that the fewer the autopsies the fewer will be the proportional numbers of cancer cases diagnosed. The authors write:

"A general review of the facts elicited on the diagnosis of cancer in London and Irish Hospitals respectively reveals the magnitude of the differences which may exist between the recorded frequency of cancer and its absolute incidence. Procedures which in London and England generally make the recorded incidence approximate nearly to the true incidence are inoperative in Ireland. The discrepancy between the recorded frequency of cancer in the hospitals of London and Ireland can be explained solely by the disadvantages under which the search for cancer is carried out in the latter country."

The authors publish some hospital statistics which demonstrate that many cases of malignant disease will be overlooked if a post-mortem examination does not form part of the inquiry. The data suggest (if we have correctly read the table on p. 14) that possibly the number recognised on clinical examination would be increased by

21% as a result of simple post-mortem examination or by 35% if the latter be supplemented with microscopic examination (312 became 377 and 338 became 456 respectively). An increase of one-third is not sufficient to bring the Irish rate (taking the proportion revealed among in-patients of hospitals) up to that of England. Simply on the evidence actually adduced, the writers' statement is, perhaps, unproven: this is by no means to say that it is false and we have referred to it here because it points to an important *possible* source of error in Maynard's data. Maynard's investigation of the influence of bad diagnosis, although evidently scientific and sufficient so far as it goes, does not, we think, go far enough. The low correlation between the cancer rate and the rate of cases ill-defined or not defined does not guarantee the accuracy of the diagnosis. Such a correlation might only indicate, if we may to make the point clear be allowed to state it rather flippantly, that the cancer rate was not affected by the conscious ignorance of the medical practitioner. A high proportion of undiagnosed causes of death may not be so much a measure of ignorance *per se* as of conscious ignorance. It is evidently of importance to determine whether there really is any appreciable difference between the amount of medical skill available in the different cities used by Maynard. How this might be expected to operate can be readily seen. Suppose we had only two cities in any district in one of which there was an active and scientific medical school and in the other no medical school or an inferior one. Not only will patients tend to flow towards the former for their own sakes, but in addition there will be attempts on the part of the authorities of the scientific school to attract cases for the purpose of study and demonstration. Both cancer and diabetes are diseases which an active school might desire to have in its wards. It is no doubt perfectly true, as Maynard remarks, that little or nothing can be done for diabetes but the disease is one of peculiar interest to chemical pathologists to the very end of life, owing to the phenomena associated with diabetic coma. The interest and importance of every phase in the life history of a person suffering from cancer are of course obvious. All these considerations suggest that an apparent correlation between the diabetes and cancer rates might very well be a secondary consequence of an association between both and the presence of efficient medical schools and we therefore specially investigated this point.

We first attempted to sort out the cities on the basis of the laws governing medical practice in the various states of the Union. These laws have been epitomised in a publication issued by the English General Council for Medical Education. A careful study of them

convinced us that we could not with their aid determine which cities were better or worse supplied with scientifically trained doctors. We then considered the state of the medical schools in the various cities. Here we had better material to use, in the shape of Flexner's searching inquiry into the condition of medical education in the United States. With the help of Flexner's descriptions we were able to classify the cities used by Maynard into those containing undoubtedly efficient medical schools and those the medical schools of which were either inefficient or non-existent. We thus had data for determining the correlation between the efficiency of the medical school and the rate of cancer or diabetes. The correlation could, however, only be determined by Pearson's two row method which assumes, (1) that the regression is linear, and (2) that the variable given only in two categories is approximately normal in distribution. Both these assumptions being of doubtful validity in the present case, the resulting coefficient can hardly be compared with others calculated from product moments in the ordinary way. The results appear in Table I.

Since evidently the correlation between goodness of medical school and height of the cancer rate might be indirect and due to the correlation of both variables with the size of the city, we desired to find the partial correlation of medical schools with cancer rate keeping population constant. But this involved the introduction of a coefficient calculated on a different basis, viz. by direct product moments, and introduced a risk of the coefficients themselves being, as it were, heterogeneous; however, the result, for what it is worth, is that the correlation between presence of a good medical school and the cancer rate for constant population is $\cdot2349 \pm \cdot1008$ (see Table I).

We think this finding is evidence that there is some correlation between the efficiency of the medical school and the cancer rate.

In order to get some collateral check we have turned to English cities and Metropolitan boroughs with populations over 200,000, and have compared the rates in cities with medical schools with those in cities without medical schools.

Both the English and American results agree in showing that there is a small but distinct correlation between the presence of a medical school and the cancer death rate, a fact which, so far as it goes, substantiates the view of the Imperial Cancer Research workers that the actual rate of cancer is influenced by the frequency and efficiency of post-mortem examinations. But the correlations we have determined decisively and we think finally negative the idea that Maynard's high

TABLE I.

Correlation between presence of a good medical school in a city and the mortality from cancer and from diabetes respectively. Males and Females.

(1) 40 American Cities¹.

Mean death-rate ² from cancer for all cities	=	732·625
Mean death-rate from cancer for 20 cities with good medical schools			=	771·500
Standard deviation of the cancer death-rates for all cities	=	128·671
Number of cities with reputable medical schools	=	20
Correlation between death-rate from cancer and the presence of a good medical school	=	+·3787 ³
Mean death-rate from diabetes for all cities	=	105·850
Mean death-rate from diabetes for 20 cities with good medical schools			=	108·950
Standard deviation of the diabetes death-rates for all cities	=	32·151
Correlation between death-rate from diabetes and the presence of a good medical school	=	+·1208 ³
Mean population for all cities...	=	374·00
Mean population for 20 cities with good medical schools			=	574·45
Standard deviation of the population for all cities	=	449·24
Correlation between population and the presence of a good medical school	=	+·5592 ³
Correlation between death-rate from cancer and population	=	+·3511 ⁴
Correlation between death-rate from cancer and the presence of a good medical school keeping the population constant	=	+·2349 ⁵

(2) 22 English Cities and Boroughs having populations over 200,000⁶.

Mean death-rate ² from cancer for all cities	=	995·36
Mean death-rate from cancer for 10 cities with medical schools...			=	1023·00
Standard deviation of the cancer death-rates for all cities	=	79·62
Correlation between death-rate from cancer and the presence of a medical school	=	+·3980 ³

¹ The corrected death-rates from cancer and diabetes used in these calculations were obtained from a paper by Dr Maynard of Pretoria entitled "A Statistical Study in Cancer Death-Rates" (*Biom.* vii. p. 276). Mr Abraham Flexner's Report on "Medical Education in the United States and Canada" was used for determining which cities contain reputable medical schools.

² Corrected death-rate per 1,000,000 living.

³ Calculated by Professor Pearson's method for cases when one variable is given quantitatively and the other in alternate categories (*Biom.* vii. p. 96).

⁴ Calculated by the ordinary product moment method.

⁵ Using the ordinary formula
$$r_{xy} = \frac{r_{xy} - r_{xz}r_{yz}}{\sqrt{(1-r_{xz}^2)(1-r_{yz}^2)}}.$$

⁶ The deaths from cancer for the different cities were kindly supplied by the Registrar General of England and Wales. The actual figures are given in Table XI.

correlations can have been seriously affected in this way. We have found that there is no appreciable correlation between the presence of medical schools and the diabetes death-rate (Table I); further, and this seems to amount to a complete demonstration of the point, if we select from Maynard's data those cities *with* good medical schools and correlate the diabetes and cancer rates for those cities alone, the result is almost exactly the same as yielded by the whole of the cities taken together (see Table II).

TABLE II.

Correlation between the corrected death-rates from cancer and from diabetes in 20 American cities all possessing good medical schools. Males and Females.

Mean death-rate from cancer = 771.50 | Standard deviation of cancer death-rates = 132.97
 Mean death-rate from diabetes = 108.95 | Standard deviation of diabetes death-rates = 34.99

Correlation between death-rate from cancer and from diabetes
 in 20 American cities possessing good medical schools = $+ .6392 \pm .0892$
 Correlation between death-rate from cancer and from diabetes
 for all the 40 cities¹ = $+ .6802 \pm .0573$

¹ Calculated from data given in Dr Maynard's paper.

We now turn to an extremely important matter, viz. the influence on the observed correlations of racial mixture. The United States of America differ from all other civilized countries in the extent to which the inhabitants are foreign born and in the numbers of different nationalities represented in their population. A simple illustration is afforded by the fact that in 37 cities, which we have specially investigated, the mean proportion of the inhabitants who were children of foreign born parents was 57 %. We have no strictly comparable data for other countries, but it seems in the highest degree improbable that a similar state of affairs prevails in any of those we have studied. Now it is readily conceivable that these conditions might exert an effect upon the correlation between diabetes and cancer even if the relation between the diseases themselves were not direct. Thus, considering two races, *A* and *B*, let us suppose that in the former the liability to develop cancer were high and in the latter the liability to die of diabetes were great. Then, if immigrants from *A* and *B* tended to pass into the same cities in about equal numbers a high correlation between the death-rates from the two diseases might be produced. The same remark would of course apply if in any given nationality both rates tended to be high; this would

happen if the two diseases were dependent on or related to the stage of culture attained by a nation.

A solution of this problem is of the first importance but materials for obtaining it do not seem to exist. We know the numbers of foreigners derived from different nations in each city, but we do not know either their separate age distributions or the death-rates from the two diseases which should be applied to them. It would be futile to use the rates obtaining in the countries of origin, since the figures are probably not valid for international comparisons.

We have only been able to approach this problem indirectly and our results are inconclusive. We adopted the following method:

The different nationalities were divided into 13 groups and we then determined for each city what Pearson has defined as a coefficient of intra-racial heterogeneity (*Biom.* 1907, v. 198). When this coefficient is high it means that the corresponding city differs greatly in racial composition from the general constitution of the whole population in the cities studied and when it is low the inference is the converse. We then ranged the cities in the order of their coefficients of heterogeneity and divided them into two equal groups—the one containing the more and the other the less divergent cities. The correlation between the corrected death-rates from cancer and diabetes was then calculated separately for each group and found not to differ significantly from that given by all the cities taken together (Table III). ($\cdot6044 \pm \cdot1009$ (less heterogeneous), $\cdot7103 \pm \cdot0788$ (more heterogeneous), $\cdot6769 \pm \cdot0542$ (all cities together).)

There was a slight diminution in passing from the more to the less heterogeneous but not a significant one. We repeated the process with a smaller group of 11 cities having smaller coefficients of heterogeneity, but once more no significant difference was revealed ($\cdot6115$).

In interpreting these results it should be remembered that the word heterogeneity is used in a rather ambiguous sense. Because a city has a small coefficient of heterogeneity it does not mean that its population is homogeneous; far from it; but merely that it does not differ markedly from the racial distribution characterising the whole "population" of cities. Had we found a significant result we should have been entitled to say that intra-national heterogeneity (*i.e.* relative heterogeneity) is a factor of importance—a failure to discover this by the present method is not, however, decisive¹. To settle the matter, details of the

¹ The point is rather *how* the heterogeneity is produced than its magnitude as revealed by the above method.

TABLE III. UNITED STATES OF AMERICA.

A. *Correlation between corrected death-rates from cancer and from diabetes in 36 American cities allowing for the racial heterogeneity of the different cities. Males and Females.*

- (1) *Correlation for 18 cities with coefficients of racial heterogeneity less than .1¹.*

Mean death-rate from cancer = 673.278		S.D. of cancer death-rates = 90.541
Mean death-rate from diabetes = 96.944		S.D. of diabetes death-rates = 27.804

$$\frac{r_c}{\bar{p}} \frac{d}{\bar{p}} = +.6044 \pm .1009.$$

- (2) *Correlation for 11 cities with coefficients of racial heterogeneity less than .02.*

Mean death-rate from cancer = 683.727		S.D. of cancer death-rates = 82.936
Mean death-rate from diabetes = 104.000		S.D. of diabetes death-rates = 28.800

$$\frac{r_c}{\bar{p}} \frac{d}{\bar{p}} = +.6115. \quad \text{S.D. } .1980^2.$$

- (3) *Correlation for 18 cities with coefficients of racial heterogeneity greater than .1.*

Mean death-rate from cancer = 784.389		S.D. of cancer death-rates = 133.365
Mean death-rate from diabetes = 109.500		S.D. of diabetes death-rates = 33.735

$$\frac{r_c}{\bar{p}} \frac{d}{\bar{p}} = +.7103 \pm .0788.$$

- (4) *Correlation for the 36 cities³ taken together.*

Mean death-rate from cancer = 728.833		S.D. of cancer death-rates = 126.800
Mean death-rate from diabetes = 103.222		S.D. of diabetes death-rates = 31.543

$$\frac{r_c}{\bar{p}} \frac{d}{\bar{p}} = +.6769 \pm .0542.$$

B. *Correlation between corrected death-rates from cancer and from diabetes for a constant proportion of foreigners in 37 American cities³.*

Mean death-rate from cancer = 731.649		S.D. of cancer death-rates = 126.211
Mean death-rate from diabetes = 103.378		S.D. of diabetes death-rates = 31.049
Mean proportion of foreigners = 57.432		S.D. of proportion of foreigners = 19.680

$$\frac{r_c}{\bar{p}} \frac{d}{\bar{p}} = +.6763 \pm .0610.$$

$$\frac{r_c}{\bar{p}} \frac{f}{\bar{p}} = +.4003 \pm .0944.$$

$$\frac{r_d}{\bar{p}} \frac{f}{\bar{p}} = +.3598 \pm .0979.$$

$$\therefore \frac{r_c}{\bar{p}} \frac{f}{\bar{p}} \frac{d}{\bar{p}} = +.6225 \pm .0689.$$

¹ For the actual values of the coefficients see Table IV.

² See footnote (3) to Table XIII (p. 111).

³ The proportion of coloured persons could not be obtained for 4 cities, so that coefficients of racial heterogeneity could only be determined for 36 cities. The proportion of foreigners was obtained for 37 cities. The data for the calculations were obtained from the 12th Census of the United States of America, 1901-1902.

C. *Correlation between corrected death-rate from cancer and from diabetes for a constant proportion of Colour and Irish in 36 American cities.*

Mean proportion of Coloured¹ = 7·6183 S.D. of the Coloured = 11·2600

Mean proportion of Irish = 10·3792 S.D. of the Irish = 6·7425

$$r_{\frac{c}{p} \frac{d}{p}} = + \cdot 6769 \pm \cdot 0609.$$

$$r_{\frac{d}{p} \frac{cl}{p}} = - \cdot 2592 \pm \cdot 1049.$$

$$r_{\frac{c}{p} \frac{cl}{p}} = - \cdot 1618 \pm \cdot 1095.$$

$$r_{\frac{d}{p} \frac{I}{p}} = + \cdot 3454 \pm \cdot 0990.$$

$$r_{\frac{c}{p} \frac{I}{p}} = + \cdot 2473 \pm \cdot 1055.$$

$$r_{\frac{cl}{p} \frac{I}{p}} = - \cdot 4344 \pm \cdot 0912.$$

$$\therefore r_{cl \ I} r_{\frac{c}{p} \frac{d}{p}} = + \cdot 6491 \pm \cdot 0650.$$

¹ See A (4) for means and S.D. of the cancer and diabetes death-rates.

TABLE IV. UNITED STATES OF AMERICA.

Corrected cancer and diabetes death-rates, coefficients of racial heterogeneity and condition of medical education in 40 American cities¹.

Cities with at least one reputable medical school. (Flexner)				Cities with either poor medical schools or none. (Flexner)			
City	Cancer death-rate	Diabetes death-rate	Coefficient ² of racial heterogeneity	City	Cancer death-rate	Diabetes death-rate	Coefficient of racial heterogeneity
Queen's	565	106	— ³	St Joseph	446	58	·064
Indianapolis	596	95	·072	Memphis	497	55	·174
Omaha	659	73	·057	Alleghany ⁴	537	59	·046
Pittsburg	686	55	·046	Scranton ⁴	561	113	·057
Washington	703	99	·184	Jersey City ⁴	627	94	·059
Cleveland	703	66	·153	Louisville	648	78	·095
Columbus	702	85	·063	Toledo	653	115	·057
St Louis	722	74	·090	Kansas City	652	70	·070
Philadelphia	723	98	·127	Denver	712	125	·050
Brooklyn	725	128	— ³	Paterson ⁴	719	100	·108
Syracuse	735	111	·036	Minneapolis	721	89	·199
New Haven	774	148	·061	St Paul ⁴	724	87	·095
Baltimore	793	101	·130	Fall River ⁴	753	131	·185
Detroit	807	122	·116	Newark ⁴	769	119	·051
New Orleans	812	65	·168	Worcester ⁴	756	132	·088
Buffalo	820	163	·117	Rochester ⁴	808	132	·053
Chicago	840	100	·216	Cincinnati	811	91	·100
Boston	937	151	·179	Richmond	816	142	·126
Manhattan	944	175	— ³	Providence ⁴	832	156	·242
San Francisco	1184	164	·121	Milwaukee	833	109	— ³

¹ Classified by means of Flexner's report.

² For method of calculation see *Biom.* v. p. 198. Data for this calculation were obtained from 12th Census of the United States of America, 1901-2.

³ Coefficient in this case could not be calculated as the necessary data could not be obtained for the particular unit used for the calculation of the death-rates.

⁴ Had no medical school.

age distribution and rates of mortality from cancer and diabetes for each of the nationalities represented in the cities would seem essential. We could then correct for both age and nationality. We also measured the effect of keeping the proportions of certain largely represented nationalities constant. Thus coloured persons and Irish are largely represented in many of the cities.

We worked out the correlation between the two rates—keeping the proportion of Irish and coloured persons constant. The coefficient is $.6491 \pm .0650$ (see Table III). No real change has been effected. It is vexatious not to be able to arrive at more definite results upon this point, since it is hard to escape from the impression that this factor of racial heterogeneity may have been extremely important in leading to the results, but with the data at our disposal it seems quite impossible to arrive at scientifically valid conclusions. The opinion may perhaps be ventured that, for the purpose in hand, the racial heterogeneity of the American cities introduces a disturbing influence which renders the data less suitable than some of those we have used.

In concluding our review of Maynard's pioneer investigation we desire to pay a tribute to its value and importance. Maynard was the first medical writer to apply exact statistical methods to the elucidation of the cancer problem and his paper might well serve as a model for those desiring to come to close quarters with this important branch of study. We shall now turn to our own investigations.

Ideal data for the study of this problem would conform to the following standards:

(1) There should be a large number of districts or towns for each of which the cancer and diabetes rates are known.

(2) The population in each district should be so large or the record should extend over so long a period of time that the actually recorded deaths may reasonably be taken as accurate measures of the prevalence of the diseases studied.

(3) It is desirable that all the populations should be large enough to fulfil the requirements of (2), but that they should not vary enormously from district to district.

(4) It is necessary that the state of medical knowledge and the organization and control of vital statistics should be such that we can regard the records as reasonably trustworthy.

(5) It is desirable that the districts used should not exhibit marked racial and economic heterogeneity.

The importance of all these considerations, except (3), is too obvious to need detailed justification. With regard to (3) the point is a statistical one. When the populations differ very greatly in size the standard deviation becomes large relatively to the mean and this introduces analytical difficulties in comparing correlations based upon absolute numbers with coefficients based on proportional frequencies (*vide infra*). We shall now consider how far our material conforms to the above standards.

We have examined most of the published vital statistics of civilized countries and have used the following :

- (1) Vital Statistics of the Swiss Confederation ;
- (2) Vital Statistics of Italy ;
- (3) Vital Statistics of England and Wales.

Contrary to expectation, neither the German nor French vital statistics are tabulated in a manner suitable for the purpose of the present research.

Switzerland.

The advantages of the Swiss material are numerous. In the first place, there are reasons to believe (we have received assurances on this point from more than one authority on the subject) that these data are from the medical point of view to be compared favourably with those of any other country. It is probable that the recorded cancer incidence differs less from the real incidence than elsewhere. In the second place, thanks to the co-operation of Mr Jessup and the courtesy of the officials of the Swiss Statistical Bureau, we have far more detailed information respecting these data than in any other case. For these reasons we should be inclined to accord to Switzerland a leading place in order of importance.

There are, however, some grave disadvantages to be set against the merits detailed. These mainly depend upon the fact that Switzerland is a very small country; we can tabulate the figures for only a small number of districts, and in addition the absolute populations of these districts are in several cases very small, only a few thousands where we should like to see hundreds of thousands. In the case of so common a disease as cancer this may not be of much importance; we might suppose that the recorded rates approximate fairly closely to the real rates and that the effects of random sampling have not been of moment although in the light of further results we are by no means confident of this. Certainly the statement is not true in the case of a rather

uncommon disease like diabetes. In that case the recorded rate depends in a few instances upon less than ten recorded deaths, in one or two no deaths are recorded at all. This means that the diabetes rates are subject to a considerable error,—addition or subtraction of a few cases would make all the difference to the calculated rate. Then again, although a small country, it is probable that Switzerland is by no means homogeneous, either racially or economically. The census of languages affords some, but by no means a complete, idea of this. Were the different racial elements uniformly scattered over the country, the heterogeneity would be of no great importance, but we can hardly assume this without an inquiry we have no means of undertaking. It will be seen then that the Swiss data are certainly not ideal figures for the study of this problem, although we do not question their utility and importance.

Italy.

In this case we escape from the difficulties attendant upon the use of a small population. The numbers are so large that even when subdivided into as many as 69 districts, we have in each case an absolute population sufficient to avoid any serious error with respect to the value of the calculated rates—so far as such error depends on the effects of random sampling. The questions of heterogeneity and the medical value of the records still remain. With regard to the former it is perhaps probable that the population is economically more uniform than is the case in industrialized communities such as England; with respect to racial elements we have no special information which enables us to offer an opinion.

So far as the accuracy of the records is concerned, we have little to go upon; in the mind of a statistician, the convenient and logical way in which the data are compiled and published creates a prejudice in their favour, but this may only be a prejudice. It should, however, be remarked that the medical authorities in this country presumably recognise that the standard demanded by the Italian Government on admission to medical practice is not greatly different from our own, since Italy is the only European country with which complete reciprocity of practice exists. But this may be little evidence as to the real condition of medical science in Italy. On the facts before us we should be disposed to regard the Italian data as on the same footing as the English.

England.

We have used the following English data:

(1) The returns for registration counties as published by the Registrar General.

(2) The records for 1911 of towns having more than fifty thousand inhabitants, specially supplied to us with full material for calculating age and sex corrections by the courtesy of Dr Stevenson.

The English counties in respect of population are quite as satisfactory as Italy and superior to Switzerland. On the other hand the economic heterogeneity of the counties taken as a whole is enormous; indeed such as to preclude any expectation of satisfactory results when they are so taken. This may be to some extent avoided by using the Registrar General's classification into urban and rural counties, but we are doubtful how far this process is really successful. When we have formed more truly homogeneous groups the numbers are very small and any attempt to enlarge the number of districts upon which the coefficients are based introduces again the heterogeneity noted.

An additional source of difficulty is the enormous range of the populations; the range in the case of the other countries and cities is indeed considerable, but far less so than in the registration counties of England. The cities do not present the same difficulties, but here we come upon the other horn of the dilemma, since the absolute populations are too small to allow us to attach very much importance to the diabetes rates which are calculated from the returns of a single year.

With respect to the scientific reliability of the certified causes of death, it is unnecessary for us to offer any observations, the question being one that each reader can answer for himself. We may note that the unreliability of the rates in the case of the English cities is somewhat mitigated by the fact that we have a large number of separate cities—118 being available for tabulation.

This general account of the data will enable the reader to compare the substratum of our work with that upon which Maynard's conclusions were based. The general conclusions which we are disposed to draw may be stated as follows:

It is doubtful whether the presumed superiority of the Swiss statistics in regard to material and scientific accuracy of the returns really compensates for the paucity of numbers. From the latter point of view both the Italian and English data will sustain a comparison with Maynard's material. It has not, however, been possible to institute

a completely valid comparison between European and American urban districts since, although our English cities are superior to Maynard's cities in point of numbers, they are inferior in size and in the validity of the rates—the latter being based upon the returns of a single year instead of on five years, as in the case of America. At the same time, were Maynard's correlation to mark an innate interrelationship of diabetes and cancer, a biological phenomenon *quod semper quod ubique quod ab omnibus* can be recognised, we should evidently expect to obtain a substantial agreement between the different series of results.

It will now be necessary to devote a few words to the question of statistical methods. As we stated above, Maynard employed either $p'cd$ or $p'c'd$ where p = population, c and d the crude deaths and c' and d' the corrected deaths. Since the value of $p'cd$ might conceivably be affected by the introduction of spurious correlation, it can further be corrected by Pearson's method. With regard to the latter correction we may say (1) that in the case in which Pearson applied it to Maynard's coefficients no change was produced; (2) it is doubtful, in our opinion, whether the correction is ever likely to produce a substantial change. We ourselves endeavoured to form some idea of the magnitude of the spurious element introduced by correcting for age distribution by means of an empirical test.

Drawings were made from bags containing different proportions of red and white counters and the "rates," i.e. the proportions of red counters, in different drawings were corrected to a "standard population." A series of coefficients was calculated and the average result led us to believe that the spurious element introduced was unlikely to be a serious source of error in practical work. It would, however, be unscientific to attach too much importance either to general ideas or to the result of a single test; we have in some cases applied Pearson's correction and have never succeeded in altering the coefficients to an extent which would affect the conclusions based upon them.

A more important matter is connected with the difference between $p'cd$ and $p'c \frac{d}{\bar{p}}$. In theory, under certain specified conditions, these coefficients should be identical and it can be proved that they are identical when the standard deviations of the various characters are small in comparison with their respective means. This condition is not fulfilled by any of our series and there is consequently a marked divergence between the two coefficients in some cases. The only instance in which the difference is such that it might affect the

reasoning based upon the correlations is that of English registration counties.

We have elsewhere recorded our grounds for believing that in cases of divergences the coefficient $r_{p'cd}$ is to be preferred¹, but, since the matter may be regarded as controversial, we give both coefficients in those cases which reveal important differences.

Some other peculiarities of the present inquiry are (1) in view of the close similarity in age distribution of both diseases it is plain that, if the crude rates are not sensibly correlated, it is improbable that the corrected rates will be. Consequently, if the correlation of crude rates is not significant, it is hardly worth while correlating corrected rates; (2) if it is desired to work with corrected rates, it is sufficient to calculate the age correction factor for one of the two diseases, since the two factors are found to be very closely correlated; (3) we have in most cases operated upon males alone, but experience suggests that it is not necessary to correct for sex distribution. As an example of this we have calculated the partial correlation between deaths from diabetes and deaths from cancer for population constant using 118 English towns, in the case of all persons and in that of males only. The corresponding figures are .3892 and .3820 (with respect to the values of these correlations it should be remarked that they are based upon crude figures uncorrected for age distribution).

ANALYSIS OF RESULTS.

Switzerland (Tables V and VI).

Taking the 25 Cantons as our subdivisions and confining ourselves to males, we first correlated the absolute numbers of deaths from cancer and diabetes respectively for constant population, and found $-.1741 \pm .1310$. If in addition to population we keep the absolute numbers of deaths of lunatics and deaths from cardiac disease constant, we reach $-.1531 \pm .1317$. Neither value is definitely significant. Using Maynard's method of correlating corrected numbers of deaths, we have $-.0337 \pm .1347$. Simply correlating the corrected rates with population constant we have $-.1533 \pm .1182$. If the rates are corrected on the bases of the deaths at ages in the subpopulations (the previous corrections were based on crude total death-rates in subpopulations and the age distributions of the same without reference to deaths at ages in the subpopulations)

¹ *Journ. Roy. Stat. Soc.* Febr. 1914.

the correlation is -0.1666 ± 0.1312 and, keeping cardiac diseases and deaths of lunatics constant, we have -0.0394 ± 0.1347 . None of these values are significant. The same material was then grouped into 23 districts by putting the two Appenzell Cantons together and the two Unterwalden Cantons together. In this case the corrected rates were correlated—the populations being kept constant, the result was -0.1416 ± 0.1322 , also insignificant. Twenty Cantons were then taken, by the

TABLE V. SWITZERLAND¹.

Correlations between the mortality from cancer and diabetes for Switzerland, based upon an average of 5 years (1901–1905).

Variables	Correlations
<i>Crude absolute numbers of deaths. Males. 25 Cantons.</i>	
Cancer and diabetes	$+0.7612 \pm 0.0567$
Cancer and population	$+0.9524 \pm 0.0125$
Diabetes and population	$+0.8303 \pm 0.0419$
Cancer and diabetes with population constant ...	-0.1741 ± 0.1308
Cancer and cardiac disease	$+0.9675 \pm 0.0086$
Cancer and lunacy	$+0.8162 \pm 0.0450$
Diabetes and cardiac disease	$+0.8170 \pm 0.0449$
Diabetes and lunacy	$+0.8662 \pm 0.0337$
Cardiac disease and lunacy	$+0.8629 \pm 0.0345$
Cancer and diabetes with cardiac disease and lunacy constant	-0.1531 ± 0.1317
<i>Corrected absolute numbers of deaths². Males. 25 Cantons.</i>	
Cancer and diabetes	$+0.7886 \pm 0.0510$
Cancer and population... ..	$+0.9696 \pm 0.0081$
Diabetes and population	$+0.8183 \pm 0.0446$
Cancer and diabetes with population constant ...	-0.0337 ± 0.1347

¹ The number of deaths from cancer and the populations of the different Cantons were obtained from "Statistique de la Suisse," *Mouvement de la Population de la Suisse*, while the number of deaths from diabetes as well as the death-rate at ages in the subpopulations for both cancer and diabetes were obtained direct from the Bureau Fédéral de Statistique.

² Owing to the different age distribution in the various Cantons, the crude number of deaths or the crude death-rates have to be multiplied by appropriate correction factors. These correction factors can be calculated by two methods, (1) when the death-rates at ages in all the subpopulations are known, and (2) when the death-rates at ages in the subpopulations are not known. For the Swiss data only could the death-rates at ages in the subpopulations be obtained and in certain cases (marked *) the correction factors have been calculated by the first method. In all other cases the second method was used. As a matter of fact the final values obtained by the two methods do not differ markedly. For a full description of the two methods see *An Introduction to the Study of Statistics*, by G. Udry Yule, pp. 223–225.

Corrected death-rates. Males. 25 Cantons.

Cancer and diabetes	-·1756 ± ·1307
Cancer and population... ..	-·3011 ± ·1227
Diabetes and population	+·1001 ± ·1335
Cancer and diabetes with population constant ...	-·1533 ± ·1182

Corrected death-rates. Males. 25 Cantons.*

Cancer and diabetes	-·1666 ± ·1312
Cancer and cardiac disease	+·2039 ± ·1279
Cancer and lunacy	-·4624 ± ·1061
Diabetes and cardiac disease	+·4538 ± ·1071
Diabetes and lunacy	+·5441 ± ·0950
Cardiac disease and lunacy	+·3765 ± ·1158
Cancer and diabetes with cardiac disease and lunacy constant	-·0394 ± ·1347

Corrected death-rates. Males. 23 Districts, the two Appenzells and the two Unterwalds being taken together.*

Cancer and diabetes	-·1529 ± ·1374
Cancer and population... ..	+·2015 ± ·1349
Diabetes and population	+·0722 ± ·1399
Cancer and diabetes with population constant ...	-·1416 ± ·1322

Corrected death-rates. Males. 20 Cantons, 5 Cantons containing towns of more than 45,000 inhabitants being omitted.*

Cancer and diabetes	-·1501 ± ·1474
Cancer and cardiac disease	+·3145 ± ·1359
Cancer and lunacy	-·4871 ± ·1150
Diabetes and cardiac disease	+·2122 ± ·1440
Diabetes and lunacy	+·3989 ± ·1268
Cardiac disease and lunacy	+·3349 ± ·1339
Cancer and diabetes with cardiac disease and lunacy constant	+·0227 ± ·1507

Crude death-rates. Males. 18 Towns with more than 10,000 inhabitants.

Cancer and diabetes	-·2407 ± ·1498
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Crude absolute numbers of deaths. Females. 25 Cantons.

Cancer and diabetes	+·7517 ± ·0587
Cancer and population... ..	+·9603 ± ·0105
Diabetes and population	+·7235 ± ·0643
Cancer and diabetes with population constant ...	+·2957 ± ·1231

Corrected death-rates. Males and Females. 25 Cantons.*

Cancer and diabetes	-·0722 ± ·1342
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omission of Cantons with towns of more than 45,000 inhabitants. Here also the correlations were inappreciable.

Lastly we correlated the rates corrected for ages (using deaths at ages in subpopulations) but without separating the sexes, and found $-·0722 \pm ·1342$.

TABLE VI. SWITZERLAND.

Means and standard deviations for the Swiss data based upon an average of 5 years (1901-1905).

Variable	Mean	Standard deviation
<i>Crude absolute numbers of deaths. Males. 25 Cantons.</i>		
Cancer ...	82·656	77·865
Diabetes...	4·776	5·003
Cardiac disease ...	128·672	133·440
Lunacy ...	5·800	6·072
Population (1900) ...	65,081·0	66,100·0
<i>Corrected absolute numbers of deaths. Males. 25 Cantons.</i>		
Cancer ...	83·161	82·129
Diabetes...	4·893	5·293
<i>Corrected death-rates¹. Males. 25 Cantons.</i>		
Cancer ...	1418·120	458·729
Diabetes...	69·496	55·885
<i>Corrected death-rates*. Males. 25 Cantons.</i>		
Cancer ...	1425·306	464·273
Diabetes...	70·135	57·581
Cardiac disease ...	1868·647	390·586
Lunacy ...	79·163	41·692
<i>Corrected death-rates*. Males. 23 Districts, the two Appenzells and the two Unterwalds being taken together.</i>		
Cancer ...	1357·581	348·180
Diabetes...	72·565	56·349
Population (1900) ...	70,740·2	54,345·7
<i>Corrected death-rates*. Males. 20 Cantons, 5 Cantons containing towns with more than 45,000 inhabitants being omitted.</i>		
Cancer ...	1473·352	502·165
Diabetes...	53·779	38·358
Cardiac disease ...	1785·658	365·184
Lunacy ...	71·376	39·629
<i>Crude death-rates*. Males. 13 towns with more than 10,000 inhabitants.</i>		
Cancer ...	1267·78	287·36
Diabetes...	100·00	38·884
<i>Crude absolute numbers of deaths. Females. 25 Cantons.</i>		
Cancer ...	84·224	82·607
Diabetes...	3·376	3·530
Population (1900) ...	67,536·7	67,147·0
<i>Corrected Death-rates*. Males and Females. 25 Cantons.</i>		
Cancer	1261·505	354·571
Diabetes	55·850	41·507

¹ Per 1,000,000 living.

* See foot-note (2) to Table V.

We have also worked out the correlation between the crude *rates* of cancer and diabetes in the 19 towns of Switzerland which contain more than 10,000 inhabitants. The result is $-.2407 \pm .1498$.

From a consideration of these results which are given with further details in the tables, it will be seen that no grouping of the material or variation in the method of calculation, *i.e.* the use of rates instead of absolute numbers, will produce a correlation coefficient which is definitely significant with regard to its probable error. Hence we must conclude that so far as Switzerland is concerned no correlation between the two rates can be shown to exist.

Italy (Tables VII and VIII).

We have analysed the Italian data in several different ways, as will be seen from the tables. As has been observed before, this material was in point of numbers probably the most satisfactory at our command. It will be seen that in the case of the 69 smaller administrative units (not one of these subdivisions contained less than 100,000 inhabitants), whether we use the method of rates or of the partials based on absolute numbers both in the case of crude or in that of age corrected figures, the result is substantially the same—there is no distinct evidence of a significant association. The utmost that can be contended is that a small positive correlation exists. When we deal with the larger units, the method of calculation is more influential on the result and if we attach more importance to coefficients based upon rates we should argue that some positive correlation exists. Even here, however, in the most striking instance, the coefficient is not quite thrice its probable error. The diminution in intensity of the correlation as we pass from larger to smaller units is what might have been predicted from Maynard's results (compare his coefficients for states with those yielded by cities), but the absolute values are strikingly different in the two cases. The 69 subdistricts are, it is to be presumed, more heterogeneous than Maynard's cities, since in our case we have mixed urban with rural areas. But it is difficult to suppose that the differences between the coefficients in the two cases can be explained entirely in this way, because the 16 provinces can hardly be more heterogeneous than the American states and are most likely less so. But, even here we find a very marked difference. We have already referred to the question of material accuracy and have admitted our inability to appraise the data from that point of view. But it does not seem reasonable to suppose

TABLE VII. ITALY¹.

Correlation between mortality from cancer and from diabetes for Italy. Males and Females.

Variables		Correlation
A. 16 DIVISIONS.		
(1) <i>Crude absolute number of deaths in 1905.</i>		
Deaths from cancer and from diabetes		+·7221 ± ·0807
Deaths from cancer and population		+·8767 ± ·0390
Deaths from diabetes and population		+·8254 ± ·0537
Deaths from cancer and from diabetes with population constant		-·0055 ± ·1686
(2) <i>Crude absolute number of deaths in 1906.</i>		
Deaths from cancer and from diabetes		+·8094 ± ·0582
Deaths from cancer and population		+·8796 ± ·0382
Deaths from diabetes and population		+·9276 ± ·0235
Deaths from cancer and from diabetes with population constant		-·0365 ± ·1684
(3) <i>Crude death-rates based upon an average of 5 years (1905-1909).</i>		
Death-rate from cancer and from diabetes		+·3356 ± ·1496
Death-rate from cancer and population		+·3685 ± ·1457
Death-rate from diabetes and population		-·0728 ± ·1677
Death-rate from cancer and from diabetes with population constant		+·3909 ± ·1429
(4) <i>Corrected absolute number of deaths based upon an average of 5 years (1905-1909).</i>		
Deaths from cancer and from diabetes		+·8328 ± ·0517
Deaths from cancer and population		+·8667 ± ·0420
Deaths from diabetes and population		+·9250 ± ·0243
Deaths from cancer and from diabetes with population constant		+·1640 ± ·1641
(5) <i>Corrected death-rates based upon an average of 5 years (1905-1909).</i>		
Death-rate from cancer and from diabetes... ..		+·3875 ± ·1602
Death-rate from cancer and population		+·2929 ± ·1542
Death-rate from diabetes and population		-·1125 ± ·1665
Death-rate from cancer and diabetes with population constant		+·4425 ± ·1356
B. 69 COMPARTMENTS.		
(1) <i>Crude absolute number of deaths based upon an average of 2 years (1905 & 1906).</i>		
Deaths from cancer and from diabetes		+·7845 ± ·0312
Deaths from cancer and population		+·8449 ± ·0232
Deaths from diabetes and population		+·8857 ± ·0175
Deaths from cancer and from diabetes with population constant		+·1458 ± ·0795
(2) <i>Corrected absolute number of deaths based upon an average of 2 years (1905 & 1906).</i>		
Deaths from cancer and from diabetes		+·7951 ± ·0299
Deaths from cancer and population		+·8542 ± ·0220
Deaths from diabetes and population		+·8867 ± ·0174
Deaths from cancer and from diabetes with population constant		+·1566 ± ·0792
(3) <i>Corrected death-rates based upon an average of 2 years (1905 & 1906).</i>		
Death-rate from cancer and from diabetes... ..		+·1900 ± ·0783
Death-rate from cancer and population		-·0536 ± ·0810
Death-rate from diabetes and population		+·2904 ± ·0744
Death-rate from cancer and from diabetes with population constant		+·2151 ± ·0774

¹ Data obtained from "Statistica delle Cause di Morte," *Direzione Generale Della Statistica*, for the years in question.

that the Italian standard is markedly inferior to the American, it may even be higher. It is, in our opinion, very difficult to believe that our failure to obtain the same or approximately the same results as Maynard's can be accounted for by shortcomings of the data. The question of statistical method, on the other hand, does not arise, as the findings of

TABLE VIII. ITALY.

*Means and standard deviations for the Italian data.
Males and Females.*

A. 16 DIVISIONS.	Variable	Mean	Standard deviation
(1) <i>Crude absolute number of deaths in 1905.</i>			
Deaths from cancer	1209·250	912·763	
Deaths from diabetes	82·625	47·809	
Population (calculated to the middle of 1905)	2,085,135	1,182,470	
(2) <i>Crude absolute number of deaths in 1906.</i>			
Deaths from cancer	1290·810	968·413	
Deaths from diabetes	87·125	51·327	
Population (calculated to the middle of 1905)	2,085,135	1,182,470	
(3) <i>Crude death-rates per 1,000,000 living. Average of 5 years (1905-1909).</i>			
Death-rate from cancer	576·862	197·180	
Death-rate from diabetes	43·809	13·355	
Population (calculated to the middle of 1907)	2,111,005	1,195,712	
(4) <i>Corrected absolute number of deaths. Average of 5 years (1905-1909).</i>			
Deaths from cancer	1358·593	995·924	
Deaths from diabetes	94·589	51·786	
Population (calculated to the middle of 1907)	2,111,005	1,195,712	
(5) <i>Corrected death-rate per 1,000,000 living. Average of 5 years (1905-1909).</i>			
Death-rate from cancer	607·688	215·753	
Death-rate from diabetes	45·665	13·412	
Population (calculated to the middle of 1907)	2,111,005	1,195,712	
B. 69 COMPARTMENTS.			
(1) <i>Crude absolute number of deaths. Average of 2 years (1905 & 1906).</i>			
Deaths from cancer	289·862	213·897	
Deaths from diabetes	19·681	16·434	
Population (calculated to the middle of 1905)	483,510	250,226	
(2) <i>Corrected absolute number of deaths. Average of 2 years (1905 & 1906).</i>			
Deaths from cancer	290·108	223·008	
Deaths from diabetes	19·912	17·192	
Population (calculated to the middle of 1905)	483,320	281,242	
(3) <i>Corrected death-rate per 1,000,000 living. Average of 2 years (1905 & 1906).</i>			
Death-rate from cancer	607·188	222·822	
Death-rate from diabetes	38·791	14·252	
Population (calculated to the middle of 1905)	483,320	281,242	

identical methods are furnished for comparison. The conclusion seems to be almost inevitable that the cause or causes which produce a high correlation between the rates in America, either do not operate or are overmastered by some other factors in Italy. It would seem that this part of our investigation strengthens that dealing with Swiss data.

England (Tables IX, X, XI, XII, XIII and XIV).

Our English data fall into two classes, viz. the large towns and the registration counties. In the case of the larger towns we were able by excluding a few very large cities which exceeded all the others in point of population by a considerable margin, to obtain a group of 118 urban communities, which contained a sufficient number of observations to allow one to form correlation tables. These tables were treated, as will be seen in the schedule of results (Table IX), by numerous methods and

TABLE IX. ENGLISH TOWNS.

Correlation between mortality from cancer and from diabetes in 118 English towns with populations between 50,000 and 375,000¹. Males and Females.

Variables	r	η	$\frac{\sqrt{N}}{67449} \times \frac{1}{2} \sqrt{\eta^2 - r^2}$
<i>Crude absolute numbers of deaths using Pearson's method of correcting for age distribution².</i>			
Deaths from cancer and from diabetes ...	+·8925 ±·0126	·9174 ·9094	1·710 1·405
Deaths from cancer and population ...	+·9423 ±·0070	·9511 ·9526	1·044 1·273
Deaths from diabetes and population ...	+·8820 ±·0138	·9003 ·9080	1·454 1·736
Deaths from cancer and from diabetes with population constant	+·3892 ±·0527	—	—
Deaths from cancer and cancer corrective factor...	-·1426 ±·0608	—	—

¹ The data, which are given in full in Table XI, were kindly supplied to us by Dr Stevenson.

² This method consists in calculating the partial correlation between the mortality from cancer and from diabetes with the cancer and diabetes corrective factors constant. As the correlation between the two corrective factors is always very high (·9984 and ·9825 were two of the values found during the present investigation) the cancer corrective factors can be used for the diabetes data, thus effecting a considerable reduction in the number of correlation coefficients to be calculated. See "On the Correlation of Death-Rates" by K. Pearson, F.R.S., assisted by Alice Lee, D.Sc., and Ethel M. Elderton, *Journal of the Royal Statistical Society*, Vol. LXXIII. p. 534.

Deaths from diabetes and cancer corrective factor	—	·1449	—	—
		± ·0608		
Population and cancer corrective factor	...	+ ·0343	—	—
		± ·0620		
Deaths from cancer and from diabetes with population and cancer corrective factor constant		+ ·2461	—	—
		+ ·0583		
<i>Crude death-rates using Pearson's method of correcting for age distribution.</i>				
Death-rate from cancer and from diabetes	...	+ ·3564	·4989	2·731
		± ·0542	·3892	1·259
Death-rate from cancer and population	...	+ ·0130	·2364	1·872
		± ·0620	·3681	2·944
Death-rate from diabetes and population	...	+ ·0031	·2166	1·744
		± ·0621	·1435	1·156
Death-rate from cancer and from diabetes with population constant		+ ·3566	—	—
		± ·0542		
Death-rate from cancer and cancer corrective factor		— ·7485	—	—
		± ·0273		
Death-rate from diabetes and cancer corrective factor		— ·4441	—	—
		± ·0498		
Death-rate from cancer and from diabetes with population and cancer corrective factor constant		+ ·0385	—	—
		± ·0620		
<i>Corrected absolute numbers of deaths.</i>				
Deaths from cancer and from diabetes	...	+ ·8799	·9048	1·697
		± ·0140	·9163	2·059
Deaths from cancer and population	...	+ ·9728	·9795	·924
		± ·0033	·9782	·829
Deaths from diabetes and population	...	+ ·8909	·9106	1·520
		+ ·0128	·9279	2·090
Deaths from cancer and from diabetes with population constant		+ ·1259	—	—
		± ·0611		
<i>Corrected death-rates.</i>				
Death-rate from cancer and from diabetes	...	+ ·0475	·3569	2·849
		± ·0619	·2644	2·095
Death-rate from cancer and population	...	+ ·1218	·2694	1·935
		± ·0529	·4466	3·460
Death-rate from diabetes and population	...	+ ·0462	·2106	1·655
		± ·0620	·4264	3·413
Death-rate from cancer and from diabetes with population constant		+ ·0438	—	—
		± ·0620		
<i>Corrected absolute numbers of deaths. Males only.</i>				
Deaths from cancer and from diabetes	...	+ ·7978	—	—
		± ·0226		
Deaths from cancer and population	...	+ ·9167	—	—
		± ·0099		
Deaths from diabetes and population	...	+ ·7625	—	—
		± ·0260		
Deaths from cancer and from diabetes with population constant		+ ·3820	—	—
		± ·0530		

allowance was made by the use of Pearson's corrective process, for the possibility of spurious correlation. The differences between the values of coefficients obtained in different ways, if not entirely negligible (that the various regressions are non-linear was proved by a special investigation which is detailed in another paper), are not sufficient to lead to difficulties of interpretation. The general result is to suggest that some degree of correlation between the rates exists, but that it is of an order wholly different from that found by Maynard. Our material, however, differs from his in two ways, one favourable to its value, the other

TABLE X. ENGLISH TOWNS.

Means and standard deviations for the English Towns, 1911.
Males and Females.

Variable	Mean	Standard deviation
Crude number of deaths from cancer	119·915	78·910
Crude number of deaths from diabetes	11·987	8·320
Crude death-rate ¹ from cancer	986·017	214·948
Crude death-rate from diabetes	98·453	37·258
Corrected number of deaths from cancer	116·780	74·103
Corrected number of deaths from diabetes	11·644	8·089
Corrected death-rate from cancer	995·339	127·099
Corrected death-rate from diabetes	95·254	32·819
Cancer corrective factor ²	·98750	·14008
Population (1911)	122,458	72,112
Corrected number of deaths from cancer. Males only. .	52·701	34·636
Corrected number of deaths from diabetes. Males only	5·841	4·143
Population. Males only (1911)...	58,501	34,751

¹ Per 1,000,000 living.

² Age distribution given in the 1911 Census was used for the calculation of the correction factors.

decidedly unfavourable. In our favour is the fact that the number of separate observations is nearly three times as great as that of Maynard. Against us is the necessity which compelled us to base the calculations on the records of deaths in a single year. The obvious objection to this is the uncertainty attaching to the individual records. The probable error is based upon the number of separate observations, *i.e.* the number of cities, but takes no account of the question as to the value of the separate records.

So far as the probable error calculation is concerned, 100 cities of 5000 inhabitants each would give, by the method we are using, the same probable error as 100 cities of 50,000 inhabitants apiece if the

TABLE XI. *Deaths from cancer and from diabetes during 1911 in 126¹
English towns with populations of more than 50,000.*

Town	Deaths from diabetes		Deaths from cancer		Town	Deaths from diabetes		Deaths from cancer	
	Males	Females	Males	Females		Males	Females	Males	Females
Barrow-in-Furness	0	4	15	25	Walsall	1	0	26	41
Bath	2	2	21	49	Warrington	0	1	26	26
Birkenhead	5	5	59	64	W. Bromwich	2	4	31	36
Birmingham ²	26	23	206	314	W. Ham	9	15	128	138
Blackburn	5	8	40	77	W. Hartlepool	5	3	11	37
Blackpool	6	5	34	47	Wigan	6	5	14	34
Bolton	7	11	69	86	Wolverhampton	4	5	48	50
Bootle	5	3	32	42	Worcester	4	3	20	40
Bournemouth	4	5	39	61	York	8	5	27	47
Bradford	19	20	141	212	Wallasey	1	6	31	59
Brighton	6	10	76	113	Darlington	1	3	27	30
Bristol ²	19	24	149	233	Stockton-on-Tees	0	2	17	22
Burnley	4	4	35	50	East Ham	4	6	53	56
Bury	2	3	16	30	Ilford	1	1	25	35
Cardiff	7	5	74	102	Leyton	2	7	50	60
Chester	1	3	16	24	Southend-on-Sea	3	9	27	43
Coventry	6	3	30	42	Walthamstow	1	1	49	57
Croydon	6	7	78	109	Gillingham	1	2	25	21
Derby	6	9	52	77	Acton	5	0	19	33
Devonport	2	5	33	37	Ealing	3	6	31	45
Dudley	4	2	17	17	Edmonton	1	0	30	29
Eastbourne	5	3	20	38	Enfield	2	4	23	26
Gateshead	7	3	47	42	Hornsey	3	4	29	71
Gloucester	2	2	21	19	Tottenham	5	7	47	65
Great Yarmouth	4	1	18	42	Willesden	4	8	39	77
Grimsby	5	4	28	32	Handsworth	2	4	28	29
Halifax	8	6	60	58	Wimbledon	5	0	18	30
Hastings	4	4	47	63	Aston Manor	3	2	26	10
Huddersfield	4	5	49	80	Swindon	1	3	17	22
Ipswich	6	4	35	51	King's Norton	5	2	19	36
Kingston-on-Hull	14	14	117	171	Barnsley	3	2	23	28
Leeds ²	31	22	191	251	Dewsbury	4	7	23	31
Leicester	11	17	81	154	Wakefield	3	0	25	46
Lincoln	4	1	36	35	Aberdare	2	2	11	24
Liverpool ²	23	31	311	392	Rhondda	6	4	37	39
Manchester ²	50	27	342	430	Battersea	11	9	76	91
Merthyr Tydfil	3	1	32	27	Bermondsey	8	6	63	56
Middlesbrough	4	5	36	43	Bethnal Green	8	3	57	64
Newcastle ²	18	13	116	145	Camberwell	14	10	124	145
Newport (Mon.)	1	4	23	47	Chelsea	3	2	37	55
Northampton	6	6	35	54	Deptford	2	6	52	75
Norwich	6	7	64	64	Finsbury	2	4	44	38
Nottingham	15	11	104	172	Fulham	9	7	80	94
Oldham	7	4	62	86	Greenwich	3	1	41	40
Oxford	2	3	30	43	Hackney	12	14	94	133
Plymouth	7	5	60	63	Hammersmith	9	9	66	64
Portsmouth	12	11	88	108	Hampstead	8	5	26	65
Preston	83	7	47	64	Holborn	5	2	27	30
Reading	9	4	32	48	Islington	14	15	152	184
Rochdale	7	6	52	63	Kensington	7	14	91	116
Rotherham	5	3	25	25	Lambeth ²	12	16	162	175
St Helens	7	4	28	32	Lewisham	13	14	77	104
Salford	17	15	105	117	Paddington	7	8	71	96
Sheffield ²	19	15	181	203	Poplar	9	6	86	65
Smethwick	5	2	18	39	St Marylebone	5	3	90	77
Southampton	6	8	62	66	St Pancras ²	7	15	122	153
Southport	5	5	27	40	Shoreditch	5	2	51	40
S. Shields	4	11	44	45	Southwark	5	9	98	113
Stockport	3	13	51	68	Stepney	10	19	137	108
Stoke-on-Trent	9	9	79	96	Stoke Newington	3	5	30	34
Sunderland	5	10	53	70	Wandsworth	11	14	121	204
Swansea	3	1	42	58	Westminster	12	5	107	104
Tynemouth	0	6	23	27	Woolwich	12	6	65	64

¹ In calculating the correlations given in Table IX only 118 towns and boroughs were used; 8 towns with populations of over 375,000 were omitted.

² These towns and boroughs with population over 200,000 and having medical schools were used for the correlation given in Table I (2).

TABLE XII. ENGLAND AND WALES.

Mean annual death-rates per million living, 1901-1910.

Age	Cancer		Diabetes	
	Males	Females	Males	Females
0-	36	29	4	5
5	18	13	10	10
10-	17	15	19	20
15-	31	27	36	27
20-	53	39	46	35
25-	109	170	59	51
35-	414	846	79	63
45-	1549	2321	160	129
55-	3904	4410	415	357
65-	6683	6658	731	574
75 and upward-	7874	7901	720	473
All ages	773	1027	103	90

TABLE XIII. ENGLISH COUNTIES*.

Correlation between mortality from cancer and from diabetes for English registration counties based upon an average of 6 years (1905-1910). Males.

Variables	Correlation
A. 41 English Counties; Rural, "Mixed," and Urban.	
(1) <i>Crude absolute number of deaths; using Pearson's method of correcting for age distribution</i> ¹ .	
Deaths from cancer and from diabetes	+·9741 ± ·0054
Deaths from cancer and population	+·9852 ± ·0031
Deaths from diabetes and population	+·9893 ± ·0022
Deaths from cancer and from diabetes with population constant	-·0265 ± ·1053
Deaths from cancer and cancer corrective factor	+·5423 ± ·0744
Deaths from diabetes and cancer corrective factor	+·5617 ± ·0721
Population and cancer corrective factor	+·6153 ± ·0655
Deaths from cancer and from diabetes with population and cancer corrective factor constant	-·2752 ± ·0974
(2) <i>Crude death-rates; using Pearson's method of correcting for age distribution.</i>	
Deaths from cancer and from diabetes	+·6635 ± ·0590
Deaths from cancer and population	-·2655 ± ·0979
Deaths from diabetes and population	-·3197 ± ·0946
Deaths from cancer and from diabetes with population constant	+·6334 ± ·0631
Deaths from cancer and cancer corrective factor	-·6445 ± ·0616
Deaths from diabetes and cancer corrective factor	-·6401 ± ·0622
Deaths from cancer and from diabetes with population and cancer corrective factor constant	+·4135 ± ·0873

* Data obtained from the *Annual Reports of the Registrar General of England and Wales* (1905-1910).

¹ See foot-note (2) to Table IX.

B. 32 *English Counties; Rural and "Mixed."*(1) *Crude absolute number of deaths; using Pearson's method of correcting for age distribution.*

Deaths from cancer and from diabetes	+ '9662 ± '0079
Deaths from cancer and population	+ '9517 ± '0112
Deaths from diabetes and population	+ '8973 ± '0232
Deaths from cancer and from diabetes with population constant	+ '8286 ± '0374
Deaths from cancer and cancer corrective factor	+ '6125 ± '0745
Deaths from diabetes and cancer corrective factor	+ '5496 ± '0832
Population and cancer corrective factor	+ '7558 ± '0511
Deaths from cancer and from diabetes with population and cancer corrective factor constant	+ '7806 ± '0466
Deaths from cancer and deaths from "other causes" ¹	+ '9026 ± '0221
Deaths from diabetes and deaths from "other causes" ¹	+ '8546 ± '0322
Population and deaths from "other causes"	+ '9826 ± '0041
Cancer corrective factor and deaths from "other causes" ²	+ '7680 ± '0489
Deaths from cancer and from diabetes keeping population, cancer corrective factor and deaths from "other causes" constant	+ '7871 ± '0454

(2) *Crude death-rates; using Pearson's method of correcting for age distribution.*

Death-rate from cancer and from diabetes	+ '6600 ± '0673
Death-rate from cancer and population	- '6151 ± '0741
Death-rate from diabetes and population	- '5617 ± '0816
Death-rate from cancer and from diabetes with population constant	+ '4821 ± '0915
Death-rate from cancer and cancer corrective factor	- '7786 ± '0470
Death-rate from diabetes and cancer corrective factor	- '7331 ± '0552
Death-rate from cancer and from diabetes with population and cancer corrective factor constant	+ '2068 ± '1141

(3) *Corrected death-rates.*

Death-rate from cancer and from diabetes	+ '2376 ± '1125
Death-rate from cancer and population	- '0516 ± '1189
Death-rate from diabetes and population	- '3293 ± '1063
Death-rate from cancer and from diabetes with population constant	+ '2339 ± '1127

C. 9 *Urban Counties.*(1) *Crude absolute number of deaths.*

Deaths from cancer and from diabetes ³	+ '9617 S.D. '0266
Deaths from cancer and population	+ '9693 S.D. '0214
Deaths from diabetes and population	+ '9965 S.D. '0025
Deaths from cancer and from diabetes with population constant	- '2020 S.D. '3391

¹ *I.e.* deaths from all causes except cancer and diabetes.² Correlation between "other deaths" correction factors and cancer correction factors is very high (+ '9938), so that again only the cancer correction factors need be used.³ As the number of observations is small (9), the usual values of the S.D. and P.E. of r are not applicable. The values given of the S.D. of r have been calculated from theformula $\sigma_r = \frac{1-r^2}{\sqrt{n-1}}$; see "On the Probable Error of the Correlation Coefficient," byH. E. Soper, M.A. (*Biom.* ix. p. 91).

(2) *Corrected absolute number of deaths.*

Deaths from cancer and from diabetes	+·9638 S.D. ·0251
Deaths from cancer and population	+·9761 S.D. ·0167
Deaths from diabetes and population	+·9914 S.D. ·0061
Deaths from cancer and from diabetes with population constant			-·1358 S.D. ·3470

D. 13 *Rural Counties.*(1) *Crude absolute number of deaths.*

Deaths from cancer and from diabetes	+·9866 S.D. ·0077
Deaths from cancer and population	+·9911 S.D. ·0051
Deaths from diabetes and population	+·9817 S.D. ·0105
Deaths from cancer and from diabetes with population constant			+·5375 S.D. ·2058

(2) *Corrected absolute number of deaths.*

Deaths from cancer and from diabetes	+·9868 S.D. ·0076
Deaths from cancer and population	+·9949 S.D. ·0029
Deaths from diabetes and population	+·9847 S.D. ·0088
Deaths from cancer and from diabetes with population constant			+·4039 S.D. ·2416

coefficients of correlation were the same in the two cases. But no one would seriously argue that the two results were really of equal value since with such a disease as diabetes a few cases wrongly diagnosed or improperly included in the local records might greatly affect the rate. To what extent these sources of error deprive our results of value we cannot say. It is obviously impossible to argue that the correlations are probably as high as in the American cities and would have so appeared if we had had a wider range of time upon which to base an average, but it is impossible with the data at our command to *prove* that this circumstance has not affected our values.

If we now turn to the results obtained from registration counties, we are faced with an almost insuperable difficulty of drawing trustworthy conclusions. Here the method of calculation makes an enormous difference to the results.

We will begin with the analysis of 32 rural and semi-rural counties. The object of considering this group separately is that from the point of view of industrial character this should be the most homogeneous group of reasonable size we can form from registration counties. This is not to say, of course, that it is homogeneous—far from it, but we can do no better without reducing the number of available districts to a value too small to render the calculations of any importance. Let us first take the results obtained when the methods adopted by Maynard and Pearson, viz. operating upon absolute numbers, are employed. We first correlated absolute deaths from the two diseases with population and cancer corrective factor constant (it was unnecessary to use more than one age correction factor since the diabetes and cancer factors were very

TABLE XIV. ENGLISH COUNTIES.

Means and standard deviations for the English counties data, based upon an average of 6 years (1905-1910). Males.

Variable			Mean	Standard deviation
A. 41 <i>English Counties, Urban, "Mixed," and Rural.</i>				
Crude deaths from cancer	313·967	406·606
Crude deaths from diabetes	41·984	50·711
Crude death-rate from cancer ¹	870·447	138·942
Crude death-rate from diabetes	118·983	21·811
Population ²	380,844	475,036
Cancer corrective factor ³	·93541	·13900
B. 32 <i>English Counties, "Mixed" and Rural.</i>				
Crude deaths from cancer	192·771	111·722
Crude deaths from diabetes	26·229	14·403
Crude deaths from "other causes"	3144·328	2257·073
Crude death-rate from cancer	898·906	132·703
Crude death-rate from diabetes	123·281	22·456
Corrected death-rate from cancer	795·625	72·188
Corrected death-rate from diabetes	112·969	15·325
Population	228,488	154,472
Cancer corrective factor	·89855	·11661
C. 9 <i>Urban Counties.</i>				
Crude deaths from cancer	744·889	686·197
Crude deaths from diabetes	98·000	83·410
Corrected deaths from cancer	833·278	813·713
Corrected deaths from diabetes	109·852	101·005
Population	922,556	753,111
D. 13 <i>Rural Counties.</i>				
Crude deaths from cancer	123·372	65·306
Crude deaths from diabetes	18·167	10·560
Corrected deaths from cancer	98·885	53·015
Corrected deaths from diabetes	14·577	8·535
Population	131,715	73,873

¹ Death-rate per 1,000,000 living.

² The populations are all calculated for the year 1907 according to the method described on pages xi and xii of the *73rd Report of the Registrar General of England and Wales* using the appropriate populations in 1901 and 1911 as basis.

³ The age distribution given in the 1901 Census was used for the calculation of the correction factors.

highly correlated; this was also true, curiously enough, when the age correction factor for deaths due to diseases other than diabetes and cancer was calculated; it was very highly correlated with the cancer correction factor). The result is a very substantial correlation. We then went a step further and introduced another variable, namely the

deaths from causes other than diabetes and cancer. This did not produce any change in the value of the partial correlation which is of precisely the same order as found by Maynard in the case of American cities ($.7871 \pm .0454$).

At first sight this would seem to prove that Maynard's finding is directly applicable to English registration counties and that the failure to obtain similar values in the case of other data should be disregarded, but a very curious peculiarity was revealed. We have above referred to the question of method and noted that in some cases the employment of a death-rate as variable instead of the absolute number of deaths makes a difference in the value of the partial correlation coefficient. We also remarked that in the case of Maynard's own material re-calculation of his results by the other method, viz. the employment of rates, did not in fact substantially modify the values of his coefficients. But in the case of these 32 counties the difference is very great. Thus if we use as variables the crude death-rate from cancer, the crude death-rate from diabetes, the population and the cancer age corrective factor, the partial correlation between the first two variables is only $.2068 \pm .1141$, a value of the same order as those obtained in the case of Italy, Switzerland and the 118 English towns. Here we do confront a case in which the nature of the inferences to be drawn depends upon the method of calculation. We may add that the somewhat elaborate calculations have been rather carefully revised and, although errors easily occur, we have some confidence that the results are arithmetically correct. The position is simple, if we trust the method of rates we shall conclude that these English counties do not exhibit a marked correlation between the diseases; if we trust the other method we shall conclude that such correlation is indeed very marked.

We do not propose to re-discuss the whole question of these two methods, which we have examined elsewhere, but we may direct attention to one point. In the tables we have collected correlations based upon various groupings of the counties using the two methods. It will be observed that the correlations based on absolute magnitudes are extremely sensitive to the inclusion or exclusion of certain counties, passing from a very high positive correlation to an inappreciable negative value. On the other hand, the correlations based on rates are much more steady; that is to say that the addition of nine urban counties to the 32 rural and semi-rural counties produces a vastly greater effect in the former than in the latter case. It appears to us that the ultimate reason of this is that, when we employ absolute numbers we

weight for size; a town or county with a million inhabitants affects the result much more than does a town or county with 100,000 inhabitants. If, as we consider to be the case, there is no reason why this preferential treatment should be accorded to the more populous districts (we are now assuming that the smallest population in our series is sufficient to justify the supposition that the rate based upon it is materially correct) it appears to follow that the method of rates is the more satisfactory instrument of research and that when the two methods point to different conclusions that inference which is warranted by the method of rates should be accepted.

We are, however, perfectly conscious that this conclusion may not commend itself to all statisticians (for instance, in successive numbers of the *Journal of the Royal Statistical Society*, Professor Karl Pearson and Mr G. Udny Yule have enunciated quite irreconcilable conclusions upon the interpretation of correlations between rates¹) and we desire to found our present conclusions so far as we can upon analytical results which do not depend upon the acceptance of any disputed or disputable point in the theory of statistics. If, then, we simply confine ourselves to the method of absolute numbers it is singular that we get so great a difference between the correlation in the case of rural and semi-rural on the one side and all the counties, including urban counties, on the other. It will be said at once that this is due to the employment of heterogeneous series, but we have some difficulty in accepting this view. It has been impossible to examine the point properly, because when we try to form still more homogeneous groups and calculate the correlations for purely urban and for purely rural counties separately, we are reduced to 9 and 13 observations respectively.

Calculating the correlation between corrected deaths (numbers) for population constant, we find in the case of the nine urban counties $-.1358$, and for the thirteen rural counties $+.4039$, these are both smaller than the coefficient based on the mixed 32, although it is doubtful whether the difference is significant.

If we take the standard deviation of the coefficients to be given by $\frac{1-r^2}{(n-1)^{\frac{1}{2}}}(1+11r^2/4n)$, and use this for determining the standard deviation of differences, it is found that the difference between the value for 32 mixed counties and that for the urban counties is nearly 2.6 times the standard deviation of the difference, while in the case of mixed counties compared with truly rural counties the difference is only 1.5 times the

¹ *J. Roy. Stat. Soc.* 1910, LXXIII. 534-9 and 644-7.

standard deviation. The former result is perhaps sufficient to render it improbable that the difference can be due to errors of sampling, but the latter is indecisive. It must, however, be remarked that, if the method based on absolute numbers be considered reliable it is significant that the more homogeneous groups (it is evident that the true rural counties are much more alike than are the 32 which grouped together give the highest correlation) yield lower correlations. To put this a little differently, we hold that the true state of affairs as regards the correlation between the two diseases we are studying ought to emerge when we analyse material as homogeneous as possible. In the case of registration counties this ideal is most closely approximated to if we confine ourselves to the rural counties. But in this case we do not in fact obtain so high a correlation as when the material is less homogeneous. Hence one is inclined to argue that the latter result may simply be due to the heterogeneity of the material. This argument is not, however, decisive because the error of sampling which arises when the data are reduced to 13 separate observations is very large and the error distribution imperfectly known so that the customary tests are inapplicable. On the other hand, it is suggestive that the coefficient deduced is of the same order as those obtained by similar methods from 118 towns, the latter being not so homogeneous as the rural counties but probably much more so than the 32 mixed areas.

All the previous reasoning is based on the assumption that the method of using absolute numbers is the correct procedure. If we take instead the coefficient based upon rates, the English results do not differ markedly from those yielded by both methods in the cases of Switzerland and Italy; there is some reason to think that the correlations are a little higher but they are nearer the continental results than the American ones. We are sure that this part of our work is that likely to give rise to the most dispute as to its interpretation, but we believe a careful consideration of the different lines of evidence will incline the reader to conclude that the correlation between the death-rates from cancer and diabetes in the case of English data is, if real, decidedly less marked than was found to be the case in American cities.

GENERAL CONSIDERATION OF THE RESULTS.

We are now in a position to discuss the general bearing of our work upon the fundamental problem stated at the outset.

In his note upon Maynard's results, Pearson wrote :

"I think therefore that the relation indicated by Dr Maynard between cancer and diabetes is a real association. It has been here discussed statistically, but no doubt it will be found eventually to have a physiological or pathological basis."

If the passage quoted means simply that Maynard's results cannot be referred to any methodological error, we are in complete agreement with it; both Pearson's analysis and our own seem to prove that Maynard's statistical method was appropriate and sufficient. We may indeed go further than this. Pearson did not specially consider the possibility of material errors; our investigation of this point has not been so complete as we could have wished, but, so far as it goes, it seems to indicate that the sources of error which suggest themselves are quite powerless to account for the coefficients Maynard obtained.

It is, however, possible to read a wider significance into the words quoted. Can it be said that we have statistical demonstration that the association between these two diseases depends upon some physiological identity either of structure or function which results in the production of cancer or diabetes? That is to say that, given two individuals, *A* and *B*, they are, in so far similar and in so far dissimilar that, given the same set of external stimuli, one will develop diabetes and the other cancer and that further, when the proportion of *A*'s in a community is high the proportion of *B*'s will also be high and conversely.

We can interpret this very general statement in a variety of ways:

(1) We might suppose that the peculiarities of *A*'s and *B*'s are inherent in their structure; that they have a physiological or pathological basis. If this were so we should expect to find that the association between the two rates was a universal phenomenon, since, by hypothesis, it depends upon structural peculiarities—part and parcel of the organisation.

It appears to us that our work is cogent, perhaps convincing, evidence against the truth of any such belief. Whatever may be said about certain details, we have not elicited any significant correlation between the disease in the cases of Italy and Switzerland and we have discovered, at the most, a moderate degree of correlation in the case of England. To explain away these results it is necessary to suppose either that (1) all our data—except the 32 mixed counties—are hopelessly vitiated by material or classificatory errors, or (2) that the methods of reduction which were valid in the case of American cities are not applicable to data obtained in Europe. We find it difficult to entertain either hypothesis and, consequently, do not think that there can

be any general physiological or pathological basis for the correlation between the two rates as found in America. This conclusion is an important one and we have been at some pains to set out the various reasons which have led us to adopt it. It is not necessary to repeat the evidence again at this place. We are, however, still faced with the fact that we have been unable to offer a satisfactory explanation of Maynard's results. His general hypothesis that the correlation is to be explained as a consequence of the conditions of modern life can, if we are correct, only be true if we suppose that the pressure of competition and other conditions attendant upon life in urban communities are markedly different in America from what prevails in Europe. This is a supposition which seems a little difficult to accept. The other important possibility is that associated with the racial mixture which, as we have proved, so definitely characterises the American cities. Our attempts to gauge the effect of this have been detailed and we have shown that the data are not adequate to permit of a really satisfactory investigation. If our analysis of the other possibly operative factors be regarded as exhaustive, we come by a process of exclusion to this last cause group. Other factors, of which we have no inkling, may play a part; we cannot go beyond our evidence and can merely record the conclusions to which a somewhat laborious analysis has led us.

These conclusions are :—

(1) The death-rates from cancer and diabetes are not universally correlated and the failure to obtain a significant degree of correlation in the case of European statistics does not appear to be due either to errors of method or insufficiency of material.

(2) In the case of England and Wales a significant degree of correlation appears to exist, but its intensity is probably very much less than that observed in the case of American cities.

(3) The correlations obtained in the American cities cannot be explained as results of inexact or inadequate statistical methods nor do they seem to depend upon material errors. The influence of racial heterogeneity may be of considerable importance, but it has been impossible to obtain satisfactory statistical proof of this.

In conclusion we have to express our thanks to Mr J. W. Brown, Assistant in the Statistical Department of the Lister Institute, for much assistance in the arithmetical work of this paper.

THE INFLUENCE OF OCEAN SPINDRIFT AND BLOWN SPRAY ON THE CHLORINE CONTENT OF INLAND GROUND WATERS¹.

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(With 3 Maps.)

Introduction.

IN works on Public Health and Water Analysis, it is a common observation that the amount of chlorine in inland surface waters near the seaboard is considerably higher than what obtains in samples from more inland parts.

While this is true, there seems to have been little attention given to a minute study of the ocean's influence on those inland waters and of the several factors which more or less modify such action.

The first endeavour to show that the sea made any change on the chlorine figure of inland waters was made by the State of Massachusetts.

It was found that the waters if unpolluted varied in their chlorine figures relatively to their distances from the sea, and as the state has a very uniform composition, the tabulation of the waters into groups of equal chlorine content by means of isochlor lines is of great service in estimating the purity or otherwise of any given sample, since if a water from a given area has over the normal chlorine, suspicion is aroused.

While the utility of plotting an area into "isochlors" has been appreciated in Massachusetts, very little in the direction of application to Public Health Chemistry has been attempted in this country.

Indeed, according to Thresh (*Water Supplies*, 1896, page 162), it is stated that the estimation of chlorine is of little value in insular countries since isochlors cannot be mapped out. Again, Somerville

¹ Dissertation for the M.D. degree in the University of Glasgow, June 1913.

(*Sanitary Science*, 1906, page 19) says "In districts remote from the sea and centres of population and land cultivation, such maps may be more or less reliable, but in this country they would be useless." The only endeavour so far made in Great Britain is that by Don and Chisholm. In their book (*Modern Methods of Water Purification*, 1st Edition, page 278) a map of Ayrshire is given showing the variation in the chlorine content of waters depending on their distances from the seaboard.

This map has been kindly lent me by the authors, and is here reproduced (Map 1). Apart from this, the present position seems to be that it is granted the ocean has an influence on waters in close proximity to it, but that so far as more remote waters are concerned such influence is of no moment or beyond measurement. Now, if the sea has an influence on waters near the shore, it is equally certain that such an influence cannot stop short at some fixed point, but must extend inland, depending on wind prevalence, contour of land, and other factors, and be accordingly measurable by analysis.

Area under Review.

For the purpose of this study that part of Scotland lying south of the river Forth was taken.

This gives a tract of land of sufficient size to show large variations in the analysis of its water samples. It is rich in geographical and geological variety, and is, moreover, an area showing appreciable differences in its meteorological conditions of rainfall, wind prevalence, etc.

Data for Thesis.

Through the courtesy of the Medical Officers of Health and Sanitary Inspectors, the water analysis figures for the various counties were utilised.

Methods.

These records were all carefully examined, and all suspicious and impure waters rejected. In this way a series of chlorine figures was obtained for each county. By referring to maps, the figures for small areas or certain places were then added together and the arithmetical mean computed. This value was taken as the normal for the place in question.



Map 1. Lines of equal chlorine content (isochlor lines) in ground water supplies.

It was found that when the series was long—that is when several values were available—the average approximated very closely to the lowest figure. Accordingly, when only two analyses were given, and the chlorine figures varied considerably, the lesser figure was taken. By reference to an area near at hand this figure was invariably found to be the truer.

With such a variable constituent of water as chlorine, liable to be increased by the slightest contamination, it was found essential, in order to minimise error, to divide waters respective of their chlorine into groups as follows:—under 1 part per 100,000, 1—1·4 inclusive, 1·5—1·9, 2—2·4, 2·5—2·9, 3 and over.

The waters, depending on the groups into which they fell, were thereafter plotted on to maps.

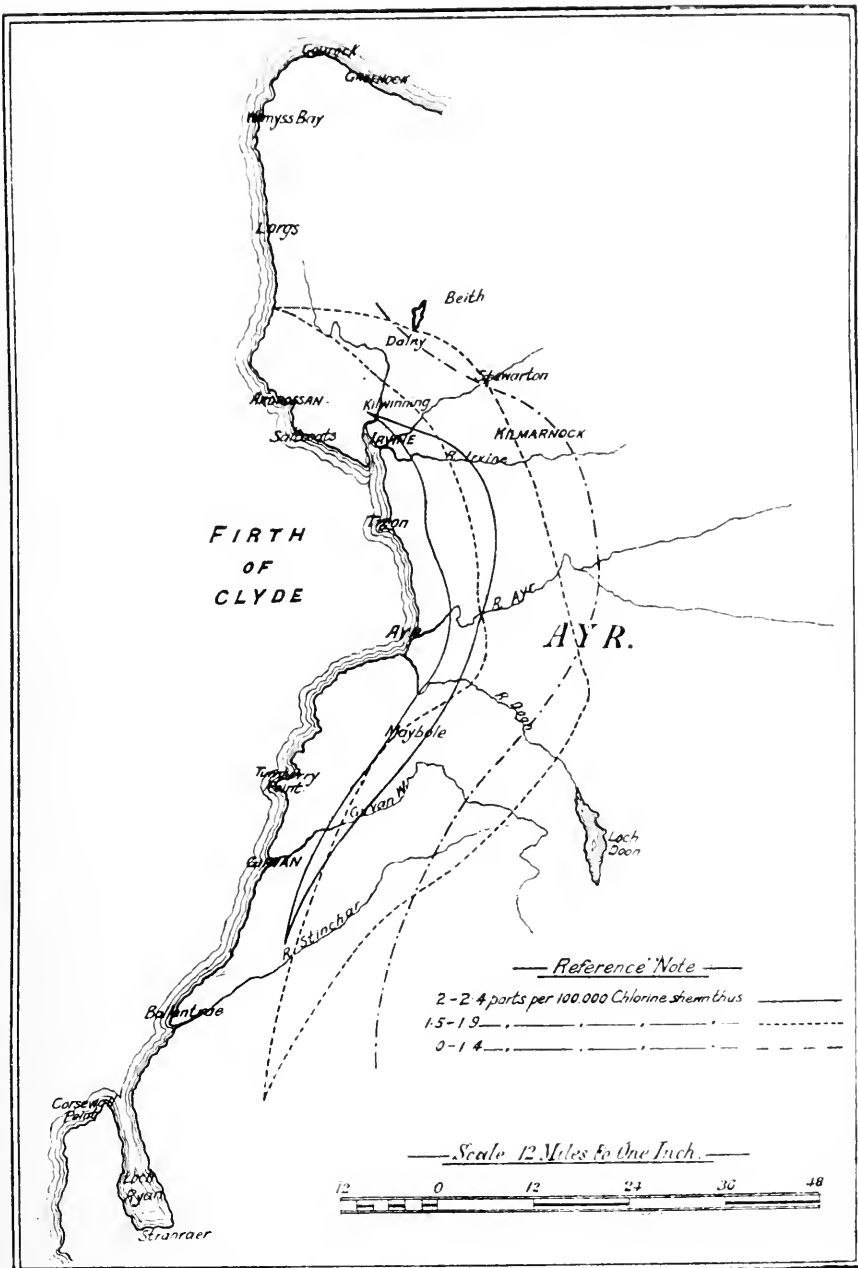
Since 1 per 100,000 chlorine was sufficient to alter any water from one group to another, great care had to be taken in drawing the isochlor lines or lines joining places of equal chlorine content. A map of Ayrshire (Map 2) shows how areas of certain chlorine values overlap and invade each other.

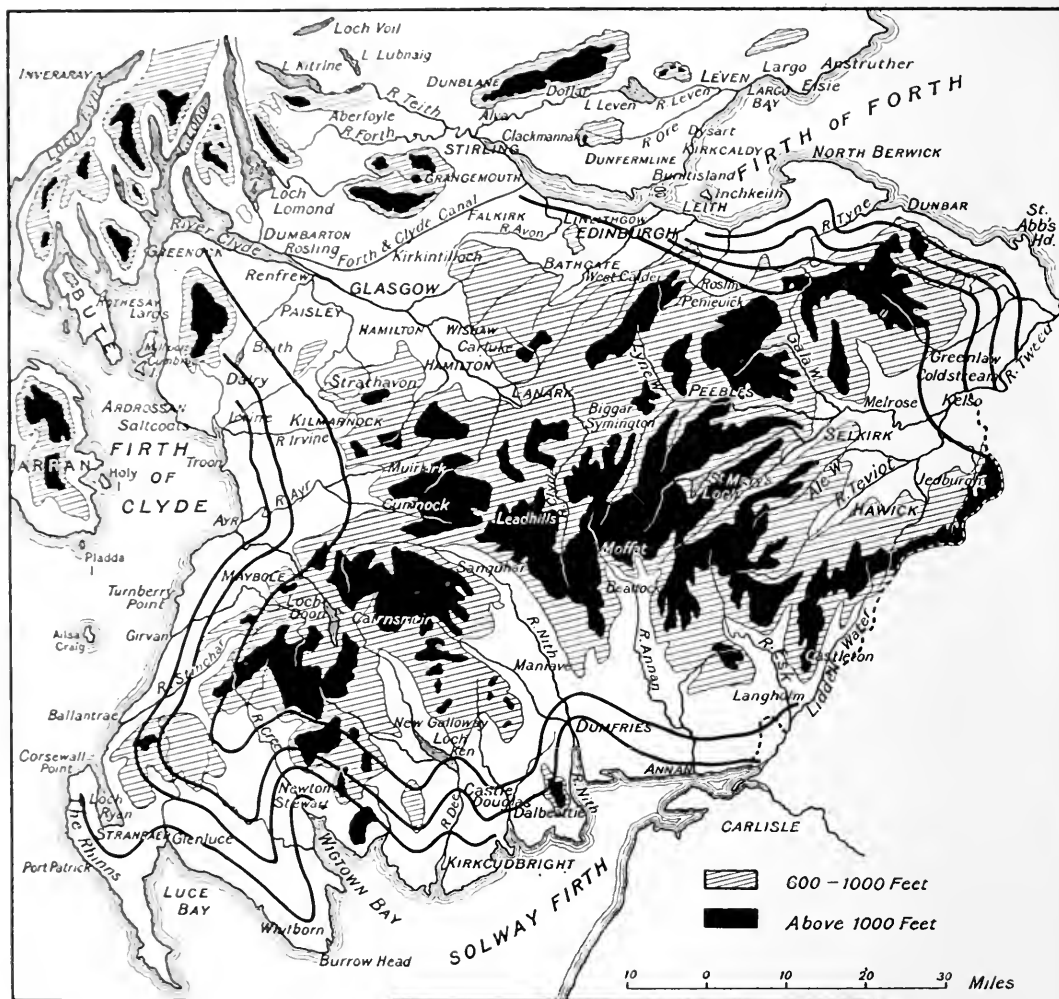
Accordingly in drawing an individual line of separation between two such areas the average had to be taken graphically.

In drawing isochlor lines there are two methods which may be termed the forward extension method and the backward extension method. In the former method points of equal value nearest the shore are joined together, in the latter points of equal value furthest from the shore.

Isochlor Lines.

In the construction of the maps the forward extension method was adopted, as it was found to show in a more convincing manner minute variations in the chlorine content. On the map (Map 3) it will be observed that the land area under consideration is divided by lines into smaller areas. These lines, drawn through places of equal chlorine content, delimit the forward extension of the given chlorine value. From the shore line to the first line inland represents that area in which the chlorine in ground waters reaches 3 and over 3 parts per 100,000. Similarly between the first and second lines the chlorine figure is 2·5—2·9 parts per 100,000.





Map 3. Areas of equal chlorine content.

The Ocean's Influence.

It will thus be seen that the chlorine content of ground waters is a diminishing one from the seaboard inland, and for this there can only be one explanation, namely, that the sea by some means or other exercises an action over inland waters.

It is commonly found that water collections on shingly land near the coast have high chlorine values due to the percolation of sea water.

This, however, can only influence surface waters within a very short distance from the shore.

Spindrift and Blown Spray.

The most potent influence the ocean has on the chlorine in inland waters is through its spindrift and blown spray. During high winds from seaward spray is carried inland in great quantity, and gets deposited over land surfaces as such.

Effect of Distance from Seaboard.

From the shore inland there is an increasing diminution in its amount due to gravitation, and consequently the amount of chlorine deposited diminishes in the same ratio as the spray does. For this reason the chlorine figure is high near the seaboard, and increasingly less as more inland parts are reached.

Effect of Land Contour.

In addition to blown spray as a factor exercising its action on the chlorine content of inland waters, minute saline particles derived from ocean spindrift are constantly producing their effect. Sodium chloride is one of the commonest constituents of the atmosphere. In greater quantity near the seaboard, being derived from ocean spindrift and blown spray, it is responsible in great part for fog and cloud production (Moore, *Meteorology, Practical and Applied*, 1910, page 197). Being particulate these particles follow the laws which govern all particulate matter, and are consequently more abundant in the lower strata of the air. In this way there is a gradual deposition of such particles from

the shore inland, with a proportionate diminution in the chlorine value of inland ground waters. The next point to be observed is that there is a deflection of the isochlor lines inland where there are valleys, or where the land is continuously low from the shore.

Effect of Wind.

In order to demonstrate this feature an orographical map has been incorporated in Map 3, showing altitudes from sea level to 650 feet, 650 feet to 1000 and over 1000 feet. By comparing this map with that showing isochlor lines it is seen to what extent the sea's influence depends on the elevation of the land surface and the presence or absence of impediment to the inward progress of ocean saline matter. Where chlorine-containing clouds are concerned, the relative elevation of the land surface is a most important factor, since on this depends in great part the precipitation of rain and the consequent liberation of the chlorine. If the land is low no precipitation occurs till high land is reached, when owing to the adiabatic cooling of the cloud, condensation takes place and there is rain. In this way the chlorine figure of waters where such conditions prevail is much higher than what obtains in the waters of the surrounding country. Since it has been shown that the sea's influence on the chlorine content of inland ground waters is exerted through the agency of ocean spindrift and blown spray, it might be expected there would be some relationship between the results of such influence and the direction of the prevailing ocean winds. For the area under consideration, however, no perceptible relationship exists. In the analysis of the ultimate results of the ocean's influence on the chlorine content of inland ground waters the factors of land elevation and wind prevalence are inseparably connected, as the former in great part assists or nullifies any action the latter may have.

Effect of the Geological Character of the Water Bearing Strata.

On comparing the isochlor map with a geological map of the same tract of country it will be noted that the individual isochlor lines show no marked divergence from their accustomed regularity on emergence from one area of certain geological formation into that of another. In works on Public Health Chemistry it is generally stated that the figure of waters varies with the geological formation of the source. As an example, the Rivers Pollution Commission of 1868 gave a table showing

the analysis of upland surface waters collected from various sources of different geological character. This Table (Thresh, *Water Supplies*, 1896, page 40), with slight alterations, is as follows:—

RIVERS POLLUTION COMMISSION, 1868.

Chlorine in Upland Surface Waters in parts per 100,000.

Geological formation					Highest	Lowest
Igneous Rocks	2.1	.31
Metamorphic, Cambrian, Silurian, and Devonian	3.3	.14
Calcareous Silurian and Calcareous Devonian	1.6	.85
Yoredale and Millstone Grits and Non-Calcareous Portion of Coal Measures	1.6	.64
Calcareous Portion of Coal Measures	4.9	.85
Mountain Limestone...	1.6	.9

It is quite erroneous to set limits to the amount of chlorine in waters from any geological formation, except where beds of salt occur. The chlorine figure is entirely dependent on the factors which have been heretofore discussed, being high when the sample has been derived from near the seaboard and low when from more inland parts.

Chlorine Figure as an Index to the Degree of Purity of Water.

Now that it has been shown that within slight margins of error the chlorine of uncontaminated ground waters of given areas approximates a normal, it would appear that the estimation of chlorine would form a rapid method of gauging for preliminary purposes the purity or otherwise of any water sample. Once the normal has been established any water showing an excess of chlorine could be classed as suspicious or impure till a fuller analysis confirmed or rejected the decision. For this purpose it would be necessary to have each district mapped out into areas of equal chlorine content by means of isochlor lines. In this way the normal could be rapidly found and the chlorine value of the sample of water under consideration compared with it.

As a result of these observations the following conclusions have been arrived at:—

Conclusions.

1. That the ocean has an influence on the chlorine content of Inland Ground Waters, an influence which is not limited to waters near the seaboard only.

2. That this influence is exerted through the ocean spindrift and blown spray deposited on the land by all forms of precipitation.

3. That this influence manifested by the chlorine in such waters varies with the distance from the seaboard, diminishing as it extends inland.

4. That this influence is modified by the configuration of the land surface being greater where the land is continuously low from the shore.

5. That to some extent this influence depends on the direction of the prevailing winds from seaward.

6. That (except where beds of salt occur) chlorine in inland ground waters is not due to the geological character of the water-bearing strata, but is derived from the sea.

7. That the chlorine figure may be utilised as a preliminary means of gauging the purity or otherwise of water samples from a district where the normal chlorine content has been established.

THE BIOLOGY OF *CERATOPHYLLUS FASCIATUS*
BOSC., THE COMMON RAT-FLEA OF GREAT
BRITAIN¹.

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IN view of its possible relation to the epidemiology of plague, a knowledge of the biology of the common rat-flea of England, *Ceratophyllus fasciatus* Bosc., is much to be desired, and therefore, at the request of Professor Nuttall, the present investigation was undertaken. The observations recorded below are mainly the result of experimental work with fleas conducted in the Quick Laboratory, Cambridge; but before proceeding to a description of the experiments it is considered advisable to give a short summary of the life-cycle of the rat-flea.

Life-cycle of the rat-flea.

The life-cycle of *Ceratophyllus fasciatus* is made up of four successive stages, viz., the egg, larva, pupa, and, finally, the adult insect, or imago.

The egg is an oval, translucent body, pearly-white in appearance, which adheres slightly to the material on which it is laid. When dead it loses its lustre and becomes tawny-yellow in colour. Its length is

¹ This paper was sent in February, 1913, for publication in *The Annual Report of the Medical Officer of the Local Gov't Board*. It is reprinted, by permission of H. M. Stationery Office, from the *42nd Annual Report to the Local Gov't Board, 1912-13* (Supplement containing the Report of the Medical Officer. To be purchased from Wyman & Sons, Ltd, Fetter Lane, London, E.C., or through any publisher. *Ed.*).

about 0.6 mm. After a somewhat variable period, depending on external conditions (*vide infra*), the egg hatches, giving rise to the larva, which escapes from the shell by means of a longitudinal slit at one end.

The larva resembles a small caterpillar, and is extremely active. It is composed of 13 segments. During the course of its growth it undergoes several moults; when complete, the adult larva expels the contents of its alimentary canal and then pupates. *The pupa* is usually surrounded by a cocoon spun by the larva and covered by minute fragments of grit, etc., adhering to it. Occasionally a cocoon is not formed, especially if the larva has been disturbed whilst pupating, or in the absence of some suitable substance to which the cocoon might be fastened. The pupa, therefore, may be either naked or covered.

Metamorphosis then takes place, and after a variable period the imago emerges from the cocoon. The pupal stage may be considered to extend from the completion of pupation to the emergence of the imago. The latter, when newly emerged, is only lightly chitinised, and its sexual organs are not fully developed. *The adult insect* may be at once recognised as belonging to the genus *Ceratophyllus* by the presence of a well-developed eye and pronotal comb. The species, *fasciatus*, is distinguished from other members of its genus, in the female, by the sinuous outline of the posterior ridge of the 7th sternite, and in the male, by the shape of the movable finger of the clasper, which is plano-convex and rather short and broad.

Although subject to considerable variation, the average duration of the various stages in the life-cycle of the rat-flea is as follows:—Egg, 7 days; larva, 60 days; pupa, 17 days; making a total of 84 days for the complete development from the egg to the imago.

Material and Methods.

The fleas used in this investigation were part of a large quantity that had been reared in the laboratory during the past three years whilst conducting experiments on the transmission of trypanosomes. They have been very easily bred in the apparatus, or a slight modification of it, recommended by the Indian Plague Commission (*vide Journ. of Hygiene*, vol. VI., pp. 421–536). Large glass jars, or boxes with glass sides, were partially filled with the refuse from rat cages. This consisted mainly of dried grain, rat excreta, gravel, straw, etc., and, for want of a better term, will be referred to as “flea-rubbish.” Usually

one or two rats were kept in wire cages resting on this rubbish and contained in the large glass box or jar. Under these conditions the fleas multiplied rapidly, and usually plenty of larvae could be obtained by searching the rubbish. Eggs were easily obtained by placing gravid females in a box containing a piece of black cloth. After the lapse of a few hours, numerous eggs could usually be found adhering to the cloth, on which they were easily seen.

Preliminary Experiment on the Individual Range in the Rate of Development.

In order to determine whether the rat-flea shows any considerable inherent variation in the rate of its development, a batch of 73 eggs, all laid on the same day, were kept together under the same conditions. Of these eggs 1 hatched in 10 days; 4 in 11 days; 25 in 12 days; 31 in 13 days; 10 in 14 days; 1 in 15 days; and 1 in 16 days. From this range of variation it is evident, therefore, that no just conclusions can be drawn from a few isolated observations, and this point has been kept in view throughout the following experiments.

Description of Experiments.

I. *Experiments with the ovum :*

- (a) The effect of various conditions of temperature and humidity.
- (b) The presence or absence of delayed hatching.
- (c) The influence of temperature and humidity on the percentage of eggs that hatched.

II. *Experiments with the larva :*

- (a) The effect of various conditions upon the duration of the larval stage.
- (b) The most favourable food.
- (c) Light reactions.

III. *Experiments with the pupa :*

- (a) The influence of external conditions upon the duration of the pupal stage.

IV. *Experiments with the imago :*

- (a) Longevity, when fed.
- (b) Longevity, when unfed.

- (c) Hosts on which it may feed.
- (d) Preference for hosts.
- (e) Copulation and oviposition.
- (f) Reaction to light and air.
- (g) Jumping powers.

1. *Experiments with the Ovum.*

(a) *The effect of various conditions of temperature and humidity.*
The effect of these conditions on the incubation period of the egg is shown in the accompanying Table (I):

TABLE I. *Incubation period of egg.*

Exp. No.	No. of eggs	Where laid and kept	Date when laid	Date of hatching	Average temp., Fahr.	Average humidity (wet and dry bulb) per cent.	Incubation period in days	
							Range	Average
1	45	Glass house in laboratory	3-7. v. 11	9-13. v. 11	73°	35-40	5-7	6
2	12	" "	5-8. xii. 11	11-14. xii. 11	70°	55	—	6
3	10	" "	30. xi. 10	6. xii. 10	70°	50	—	6
4	about 48	Incubator under bell-jar	—	—	90°	100	—	All shrivelled up
5	44	Open vessel in laboratory	29. iv-2. v. 11	8-12. v. 12	59.5°	66	8-10	9
6	6	" "	Summer, 1909	—	—	—	5-9	7
7	10	" "	"	—	63°	70	5-6	5½
8	2	" "	"	—	—	—	—	7
9	74	" "	30. xi. 11	10-16. xii. 11	59°	82	—	13
10	62	Engine room	13. v. 11	19-21. v. 11	58°	60-65	6-8	7
11	51	Cellar	11. v. 11	18-21. v. 11	57°	72	—	8
12	7	"	1-2. xii. 11	13-14. xii. 11	55°	75	—	12

Hatching occurred most rapidly (in 5½ to 6 days) at 63° to 73° F., the atmospheric moisture varying between 35 and 70 per cent. Hatching occurred on the 7th, 8th, and 9th days at 57° to 59.5° F., the atmospheric humidity ranging between 60 and 72 per cent. Hatching was delayed to the 12th and 13th day at 55-59° F., the humidity ranging between 75 and 82 per cent., and hatching did not occur when the eggs were maintained at 90° F. in a saturated atmosphere.

(b) *The presence or absence of delayed hatching.* In the case of the body-louse, *Pediculus vestimenti*, Warburton (1910) has observed that under certain conditions the hatching of the egg may be considerably delayed. Accordingly, the possibility of such a property being present in the eggs of fleas was carefully investigated. In many cases, eggs

were observed for periods up to two months after they had been laid, but whenever the incubation period was unduly prolonged the eggs were found to be dead. Therefore, the presence of delayed hatching has not been observed in our experiments with *Ceratophyllus fuscatus*.

(c) *The influence of temperature (57–73° F.) and humidity (35–72 per cent.) on the percentage of eggs that hatched* appears to be quite insignificant, as shown in the following experiments made to determine this point:

Number of experiment	Number of eggs laid	Temperature: Degrees Fahrenheit	Humidity percentage	Percentage of eggs that hatched
1	55	59.5	66	80
2	80	58	60–65	75
3	73	57	72	70
4	64	73	35–40	70

It will be noticed that the temperature and humidity conditions during experiments 3 and 4 were very different, and yet the percentage of eggs which hatched out was exactly the same.

In conclusion, it may be said that the egg hatches out after a relatively short incubation period (5–14 days), the duration of which depends mainly on the degree of humidity. The incubation period is never abnormally prolonged, as in the case of lice, and varying conditions of temperature and humidity have practically no effect on the percentage of eggs which ultimately hatch.

II. *Experiments with the Larva.*

(a) *The effect of various conditions.* Unfortunately, out of the larvae which were kept under observation, very few lived throughout the whole stage and pupated, and in consequence our results are somewhat imperfect.

Usually, the larvae were placed in Petri dishes, each of which contained some particular food-stuff. They were then kept in the dark under varying conditions of temperature and humidity. The results are given in the accompanying Table (II).

From these results it will be seen that a low temperature combined with a high degree of humidity is more favourable to the longevity of the larvae than the opposite condition. (Compare Experiments 3, 5, 8, and 9 with 4 and 6.) Although none of the larvae which were fed on powdered rat-faeces managed to reach the pupal stage, we do not consider that this lack of success was due to the food—a point of view

TABLE II.

No. of Exp.	No. of larvae	Temperature and humidity	Locality	Additional notes	Results
1	3	Summer, 1910	Laboratory cup- board	In a Petri dish with a little rub- bish at the bot- tom	Pupated after 52-60 days.
2	1	"	"	In watch-glass, with a feather covered with dried blood.*	Lived 39 days.
3	10	54° F. & 74 % ₀	Cellar ...	In pill-box, with powdered rat- faeces as food.	Lived 30 days.
4	10	70° F. & 50 % ₀	Glasshouse ...	" "	Lived 7 days.
5	44	59° F. & 65 % ₀	Laboratory cup- board	" "	Lived 20 days.
6	45	73° F. & 40 % ₀	Greenhouse in laboratory	" "	Lived 7 days.
7	62	59° F. & 64 % ₀	Engine-room ...	" "	Lived 4 days.*
8	51	58° F. & 72 % ₀	Cellar ...	" "	Lived 20 days.
9	49	60° F. & 83 % ₀	Laboratory cup- board	" "	Lived 30 days.

* Put into direct sunlight.

which is supported by the experiment described under the next heading (*b*). The reason that so few of the larvae ever came to maturity in these experiments was that they were usually placed in a glass dish containing only a small quantity of dry food, and nothing in the nature of a substratum of rubbish in which they might bury themselves. In the only experiment (1) in which a substratum was provided the larvae successfully reached the pupal stage. It may be added that when breeding fleas in boxes the larvae are always more abundant in those parts of the rubbish that are slightly damp. The conditions which seem to be suitable for the larva resemble those that are most favourable to the imago, for the adult unfed flea will live a very much longer time amongst hygroscopic rubbish than in its absence.

To summarise, the most favourable condition for the larva is a low temperature combined with a high degree of humidity; further, the presence of rubbish, in which the larva may bury itself, is essential to its successful development.

(b) *The most favourable food.* When larvae are placed in a bottle containing either wood-wool soiled by excrement, or with feathers or filter-paper covered with dried blood, they will thrive readily and pupate. An experiment was made to see whether dried blood or rat-faeces is most favoured. About 50 newly-hatched larvae were placed in a pill-box containing, on one side, filter-paper covered with dried blood, and on the other side, powdered rat-faeces. The larvae were found to wander from one to the other indiscriminately. The larva possesses the curious habit of always devouring its moulted skins.

(c) *Light reactions.* The larva is very sensitive to light, especially in its younger stages, and exposure to direct sunlight is rapidly fatal. Newly-hatched larvae invariably hide themselves away from the light, creeping underneath any dark objects at the bottom of the dish. Nevertheless, full grown larvae, if kept in the light, will manage to pupate.

To summarise the foregoing observations on this stage of the life-history, it may be stated that the larva is very susceptible to external conditions, and is not very "hardy." It readily feeds on flea-excrement, dried blood, and powdered rat-faeces. It is photophobic, especially in its younger stages.

III. *Experiments with the Pupa.*

The influence of external conditions on the duration of the pupal stage. The following experiments have been performed in order to determine the duration of the pupal stage under various external conditions:

1. A pupa, formed during July, 1910, was kept in a dark cupboard in the laboratory; the imago emerged after 17 days.

2. A batch of 30 to 40 "naked" pupae (*i.e.* without cocoons) were under observation for four to five weeks before the imagines emerged. The time of the experiment was April and May, 1910.

3. In October, 1911, a batch of freshly-formed cocoons was placed in a small dish that was kept near a white rat in a deep glass jar in the laboratory. Two months later one small and feeble flea was found on the rat, but no more of them emerged until February—a period of four months. Eight of the cocoons were then dissected, and seven of them were found to contain the imago, which was fully formed, but in a resting state. The remainder of the batch were then placed in the warm glass house of the laboratory (70° F.) for one night near a white rat; the next day all the cocoons were empty, and the fleas were found on the rat.

From these experiments it is apparent that the temperature greatly influences the duration of the pupal stage. Moreover, when metamorphosis is complete a low temperature will cause the imago to remain within the cocoon.

IV. *Experiments with the Imago.*

(a) *Longevity, when fed.* One experiment was performed in order to determine the longevity of fleas when regularly fed on blood.

A number of fleas were fed through gauze on a rat at various intervals for two months. During this period the insects remained alive and healthy, frequently copulating and laying eggs. The experiment was then discontinued. It is evident, therefore, that, even when sexually mature and ovipositing, the flea does not possess a short life.

(b) *Longevity, when unfed.* The experiments performed in order to determine the longevity of fleas when unfed fall into two groups, according as to whether or not the insects were kept on a substratum of more or less hygroscopic rubbish. In every case many hundreds of fleas were used.

The result of keeping unfed fleas in the presence of hygroscopic rubbish is shown in the following Table (III):

TABLE III.

No. of Exp.	Locality temp. and humid.	Quantity of substratum	Result
1	Cellar passage	Small amount	Many alive after 2 weeks; only one or two after 9 weeks.
2	Laboratory (60° F. & 70%)	Large amount	Large numbers alive after 17 weeks (December, 1910), and also after 7 months (March, 1911): but during the hot summer of 1911 very many died. On the return of the winter months many were still alive and active up to February, 1912, after being without food for 17 months. They were then accidentally thrown away.
3	Greenhouse (70° F. & 45%)	Large amount	All died within 4 months.
4	Cellar	Small amount, but more than in Exp. 1	All died within 4 months; but many were alive after 3 months.

To make certain that the continued presence of the fleas was due to the persistence of the original insects and not to a constant accession of freshly-hatched ones, the following experiments were performed:

(1) Some of the rubbish in which the fleas were living was put into a Petri dish and all imagines carefully removed. The dish was then kept under close observation for 4 months, but no fleas ever appeared in it.

(2) Some of the imagines were isolated but did not lay any eggs, nor were larvae ever noticed in the flea rubbish. It is evident, therefore, that no active breeding was taking place, and the presence of fleas must be attributed to the persistence of the original individuals.

On the other hand, the result of keeping unfed fleas in the absence of any substratum of rubbish is well shown in the following Table (IV) :

TABLE IV.

No. of Exp.	Locality	Temperature	Humidity	Substratum	Result
1	Cellar passage ...	—	—	Nil	Died after 31-38 days.
2	Cellar ...	—	—	Nil	Died after 25-32 days.
3	Laboratory ...	—	—	Moist silver sand	Died after 32-43 days.
4	Greenhouse in Laboratory	70° F.	40 %	Nil	Died after 25-31 days.
5	Incubator ...	77° F.	—	Nil	Died after 1-3 days.
6	Incubator ...	77° F.	—	Moist silver sand	Died after 1-2 days.
7	'Temperate Pit,' Botanic Gardens	60° F.	60 %	Nil	Died after 27-38 days.
8	'Stove Pit,' Botanic Gardens	65° F.	57 %	Nil	Died after 27-38 days.
9	Cellar ...	55° F.	80 %	Nil	Died after 17-24 days.

From the results of these two series of experiments it is evident that if the adult flea does not obtain food in the form of blood, its length of life depends mainly on the nature of its surroundings. In the presence of rubbish, in which it can bury itself, the flea may live for many months, whereas in the absence of any such substratum it very rarely lived a month. In the former case the length of life is influenced to some degree by the temperature and humidity, for in Experiment 3 (Table III), carried out at 70° F. and 45 per cent. humidity, the fleas did not live for more than four months, whilst in Experiment 2, with an average temperature of 60° F. and 70 per cent. humidity, they lived for at least 17 months. A low temperature combined with a high degree of humidity appears, therefore, to be most favourable to the prolongation of the life of this insect. In this connection, it may be pointed out that in Experiment 2 (Table III), it was very noticeable how large a number

of fleas died off during the summer, whilst in the winter months no diminution in numbers could be detected.

When the fleas are kept without any rubbish, the conditions of temperature and humidity seem to have little effect on the length of life (Table IV).

A few experiments have been performed with the object of explaining why fleas will live in a mass of rubbish for such a long time without any food. In order to determine whether the insect actually sucks up moisture from surrounding objects, several fleas that had been without food for several months were placed on filter-paper that had been moistened with a solution of neutral-red. The fleas were afterwards dissected, but no trace of colouring matter was found in their intestines.

It has also been observed that when fleas in bell-jars are supplied with an extract of flea-rubbish on filter-paper, they do not live any longer than those which are not thus supplied. It appears, therefore, that fleas do not suck up moisture from surrounding objects.

We suggest that the flea probably survives by creeping into husks of grain, or under pieces of straw, in which situations it is not only cool, but also supplied with a certain degree of moisture favourable to its persistence.

(c) *Hosts on which it may feed.* In addition to its normal host, the rat, *C. fasciatus* will feed on a variety of animals. As, however, Tiraboschi (1904), and Strickland and Merriman (1912) have previously given lists of animals on which this flea has been found, we shall proceed directly to a description of the experiments. In addition to determining whether the flea would feed on any particular host, the insects have been kept under observation to see if they could breed on such animals.

1. *Experiments with Man.*

Experiment 1	27/11/11	One <i>C. fasciatus</i> taken from a wild rat.
	28/11/11	Refused to feed on two men (E. H. and G. M.).
	29/11/11	Fed on G. M.
	1/12/11	Fed on C. S.
Experiment 2	1/12/11	Two <i>C. fasciatus</i> put on man (E. H.); fed.
Experiment 3	1/12/11	Three <i>C. fasciatus</i> , starved for a fortnight, fed readily on G. M.
Experiment 4	6/12/11	Eight <i>C. fasciatus</i> , starved for many months, fed on C. S.
Experiment 5	6/12/11	Five <i>C. fasciatus</i> , starved for many months, fed on C. S.

From the foregoing results it is evident that *C. fasciatus* will readily feed on man, and in this respect we confirm the work of Chick, Martin and Rowland (1911). On the other hand, fleas that had fed only on human blood could never be induced to lay eggs, and therefore it is probable that *C. fasciatus* would not be able to become established if it could only feed on man. Several fleas were fed for four successive days on C. S., but although the insects were kept under observation for some time, no eggs were laid.

On the other hand, a number of the same lot of fleas that were fed on a rat at the same time, laid eggs shortly after the meal.

2. *Experiments with a Rabbit.*

A rabbit was put into a flea-box containing a large number of hungry fleas and taken out after a few hours. The majority of the fleas were then found to be gorged with blood, evidently having fed on the rabbit. Several of the gorged females were then placed in a Petri dish, but no eggs were laid, although some of the same lot of females when fed on a rat at once laid eggs.

3. *Experiments with the Mouse.*

A large number of fleas were placed on a mouse. Although they readily became gorged on this host, none of the females laid any eggs. From these experiments it appears that, although the rat-flea will readily suck the blood of various animals, yet the rat is the only true natural host, as in no other case was the meal followed by oviposition.

(d) *Preference for hosts.* It is generally assumed that *Ceratophyllus fasciatus* prefers its normal host, the rat, to any other animal; but the following experiments do not support this supposition, at least, as far as man is concerned.

A long glass tube, about 2 inches in diameter, was taken, and at the middle of its length was placed a batch of fleas contained in a small tube. One end of the glass tube was then applied to a rat and the other to the bare arm of a man. As the fleas escaped from the small tube they nearly always went towards the bare arm and fed on human blood. The experiment was then repeated with the positions of the two hosts reversed, but exactly similar results were obtained.

In the first case, eight fleas went towards the man and one to the rat, whilst in the second, five fleas went to the man and one to the rat. It

is evident, therefore, under these conditions man is much more attractive to the flea than the rat¹.

(e) *Copulation and Oviposition.* A certain number of experiments and observations were made to determine the effect of various conditions on these two processes.

(1) Fleas just hatched were allowed to feed on a rat for successive days up to a week later, but the insects were never observed *in copula*.

(2) Fleas found *in copula* were placed in a Petri dish in the laboratory, and within 24 hours several eggs were laid.

(3) Fleas which were kept without food for long periods in order to test their longevity were never observed to copulate nor lay any eggs, although many of the insects were isolated and kept in a Petri dish.

(4) A batch of fleas which had been starved for a month and were not laying eggs were put on a rat for 24 hours and then taken off and placed in a Petri dish. The next day several eggs had been laid. The fleas were then removed to another Petri dish without having another feed, but no more eggs were laid.

(5) Fleas were fed through fine gauze on rats, and copulation immediately took place between several couples. We have often observed that, as soon as fleas are fed, the males wander about and copulate with the females, even while the latter are still sucking blood. The frequency of copulation seems to depend on the number of times the fleas are fed.

(6) A batch of fleas that had been starved for many months was placed on a rat for 24 hours, and within the next 24 hours many eggs were laid.

(7) During December, 1911, a batch of fleas was placed on a rat in a cold cellar, the average temperature of which was 50° F. and its humidity 80 per cent. These fleas when put into a dish laid eggs, and in addition larvae were seen in the rat's nest on February 15th—about two months later.

(8) A batch of fleas were repeatedly fed through gauze on a rat for a period of two months, during which time they frequently copulated and laid eggs. The experiment was then discontinued.

On summarising the above-described experiments, it appears that fleas do not copulate until they are sexually mature, and that for some time after emerging from the pupa their reproductive organs are still

¹ It is possible that in these experiments the fleas were influenced by a difference of temperature, as shown by Howlett (1910), in the case of mosquitoes.

imperfectly developed. When mature, copulation is always followed by oviposition within a very short time. Copulation takes place soon after the fleas have fed on their true host—the rat—but not if they have fed on a facultative host, such as man. In the absence of food the fleas are never observed to either copulate or lay eggs.

The effect of the rat's blood on the female with regard to egg-laying seems to be rather stimulating than nutritive, as fleas that have been without food for many months will lay eggs immediately after they are fed. Similarly, the male requires the stimulus of a meal of rat's blood before it displays any copulatory activities.

Both of these processes will take place in a rat's nest during the winter months, and therefore the temperature does not appear to be a very important factor.

In conclusion, the sexual life of the flea certainly lasts for at least eight weeks.

(f) *Reaction to light and air.* The fleas were always to be found hiding under dark objects and very seldom seen in the light, which is evidently much disliked by them. Moreover, they are very sensitive to air-currents, for when a number of fleas are blown upon they at once become very agitated.

(g) *Jumping powers.* The average flea is able to jump a distance of about 3 inches, when gorged, and about 4 inches when starved. It is also capable of walking up a vertical sheet of glass for about 8 inches, after which it falls back to the ground.

Summary of Conclusions on the Life-history of the Flea.

Our conclusions with regard to the above-described experiments have been given in detail at the end of each section, but we here append a short and condensed summary of the main features :

1. The duration of the various stages is very variable even under the same conditions.

2. Temperature and humidity are the two conditions which have most influence on the duration of the various stages. On an average, the egg hatches out in 5–14 days, an increase of humidity having a retarding, and a moderately high temperature a slight accelerating effect. The larva is soon killed by a high temperature (70° F.) combined with a low degree of humidity (40 per cent.). However, under these conditions, the larvae will live longer if rubbish is present, for they are then able to bury themselves in it and thus obtain a certain amount of

moisture. The pupal stage is much prolonged by cold, but this is partly due to the non-emergence of the imago even when it is fully formed.

The imago, at least when unfed, dies off much more rapidly in summer than in winter. Eggs are laid by the imago even at comparatively low temperatures (50° F.).

3. The larva and imagines like to bury themselves in rubbish, and under these circumstances their duration of life is much prolonged, even when other external conditions are somewhat severe.

4. When copulating, the imago frequently lives at least two months, but it will not copulate unless it is fed on rat's blood. It feeds readily on man and many other animals, but will not copulate after feeding on these facultative hosts, even though at least one of them—man—seems to be more attractive to it than its normal host, the rat. Oviposition invariably takes place within 24 hours of copulation, even when the insect has only been fed once after being starved for a period of many weeks. The rat's blood, therefore, probably contains some substance that possesses a stimulating effect on the flea's sexual organs. When starved, the imago will live for a very long time—at least 17 months—but only in the presence of rubbish in which it can bury itself. In the absence of rubbish the flea will only live for about a month, even under the most favourable conditions of temperature and humidity.

5. From the foregoing observations it is clear that the presence of rubbish containing organic matter is essential for the development of the flea.

We are greatly indebted to Professor Nuttall for his kind help and advice in this work, and for many suggestions which were made use of in the experiments. Our thanks are also due to Dr E. Hindle for his careful revision of this paper.

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ON THE CONDITIONS UNDER WHICH DISCONTINUOUS EVENTS MAY BE EMPLOYED AS A MEASURE OF CONTINUOUS PROCESSES, WITH ESPECIAL REFERENCE TO THE KILLING OF BACTERIA BY DISINFECTANTS.

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It very frequently happens in the investigation of life-phenomena that the uninterrupted progress of underlying processes is evidenced to our senses by a series of intermittent events. Thus the underlying and undoubtedly continuous processes which determine the heart-beat are evidenced to our senses by a series of intermittent pulsations; the effects of previous sensory stimulation upon our central nervous system are usually recognised by the recollection of a series of images which appear to us to be discrete and unitary in character; the continuous effects of light upon non-sessile heliotropic organisms are evidenced by their separate and unequal movement towards the source of illumination, and the action of a disinfectant upon bacteria is evidenced by a series of deaths, each individual death constituting an indivisible unit.

No especial consideration is required in order to appreciate the fact that the frequency with which any of the above discontinuous events occur affords some sort of indication of the extent to which the underlying and determining processes are taking place. It is not by any means so obvious, however, that the number of these discontinuous events is a reliable *quantitative* measure of the progress of the underlying processes. If indeed it be so, then a knowledge of that fact and of the limiting conditions under which this method of measurement may be

safely applied must be of paramount importance to the biologist, since the method opens up to him the possibility of a quantitative estimation of innumerable life-processes which would otherwise be inaccessible to measurement.

In a series of communications to this Journal H. Chick (1908–1912)¹ has confirmed and very greatly extended the observation of Madsen and Nyman (1907)² that if a uniform culture of bacteria be exposed for varying periods of time to the action of a relatively large amount of disinfectant (so that the concentration of disinfectant does not appreciably alter throughout the process), the relationship between the number of bacteria killed and the time of exposure is that which is characteristic of a mono-molecular reaction, that is, of a reaction in which only one molecular species is undergoing appreciable changes in concentration. They interpret³ this fact to mean that the underlying process which determines the death of the bacteria (*e.g.* combination of the disinfectant with some protein within the bacteria) is a chemical reaction involving a concentration-change in one molecular species. It is obvious that the validity of this conclusion depends upon the validity of employing the discontinuous events afforded by the deaths of the bacteria as a measure of the extent of the continuous underlying chemical changes *within* the bacteria.

G. Udny Yule (1910)⁴ has pointed out that the death-rate in these experiments *cannot be selective*, in other words that the successive deaths cannot be attributed to inequalities in the susceptibility of the bacteria, for otherwise the percentage-mortality would *decrease* with time as the weaklings were weeded out, whereas the results above-quoted show that the percentage death-rate is *constant*, just as the percentage of change is constant in a mono-molecular reaction. On the other hand he finds difficulty in accounting for the results on the supposition that the action of the disinfectant upon the bacteria is *gradual* and *cumulative*, as one must necessarily assume it to be if it really consists in a chemical process.

Starting with the assumption that the chance ($= p$) of an “unfavourable” change occurring in any one of the bacteria (such as the combination of a molecule of disinfectant with a molecule of the proteins which it contains) is constant for all periods of exposure. Yule

¹ H. Chick. *Journal of Hygiene*, (1908) VIII. 92; (1910) x. 237; (1912) XII. 414.

² Madsen and Nyman (1907). *Zeitschr. f. Hyg.* LVII. 388.

³ As far as we know Madsen and Nyman offered no interpretation of the facts they brought forward. (Ed.)

⁴ G. Udny Yule (1910). *Journal of the Royal Statistical Society*, LXXIII. 26.

finds that the law of mortality observed by Chick cannot be accounted for except upon the supposition that a *single* "unfavourable change" is fatal,—a supposition so inherently improbable that it may be dismissed without further consideration. If, however, we examine the actual implications of Yule's fundamental assumption that p is constant, we find that it is equivalent to assuming *that the underlying process which determines the death of the bacteria is not a chemical process*, for it is a fundamental characteristic of the time-relations in chemical processes that the frequency with which units of change occur (and therefore the probability of their occurrence) does not remain constant as a reaction proceeds but, on the contrary, varies in accordance with laws which are definite and dependent upon the number of molecules which participate in undergoing a unit of change. Thus, if the reaction be mono-molecular, the probability ($=x$) of a unit of change taking place in time $=t$ is given by:—

$$\log \frac{A}{A-x} = \kappa t$$

where A and κ are constants, and the probability of a unit of change taking place in a *unit* of time is given by the value of $\frac{dx}{dt}$ in the equation:—

$$\frac{dx}{dt} = \kappa (A - x),$$

the constant A expressing the initial mass of material subject to change.

Hence, what Yule actually proves is that *if the underlying process which determines the death of the bacteria is not a chemical process, or at least a process of which the velocity varies as it proceeds, then the quantitative results obtained by Chick are inexplicable.*

The question still remains, however, to what extent we may rely upon the quantitative results obtained by Chick and by Madsen and Nyman as *quantitatively defining* the processes which underlie disinfection. This question may be answered in the following way:—

Let x be the number of units of the underlying change which has taken place in a given time t in all of the bacteria taken together. These units of change will be distributed fortuitously among the different bacteria, so that in a certain number of them 0 units of change will have occurred, in others 1 unit, in others 2 units, and so forth. The fortuitous character of the distribution arises from the fact that in order that a unit of change may occur in any one of the bacteria it must first of all receive (collide with) a molecule of the disinfectant, and the

collisions between disinfectant-molecules and bacteria are necessarily fortuitous.

If there be N bacteria in all which are exposed to the action of n molecules of disinfectant, and they do not cease at any time to be exposed to the action of the disinfectant¹, then there are n ways in which a collision may occur with any one of the bacteria, and the chance of any given one of the bacteria receiving a single collision is $\frac{1}{N}$ and the chance of its failing to do so is $\frac{N-1}{N}$. Hence the number of bacteria which will have undergone 0, 1, 2, r , units of change ("successes") when the total number of units of change which have occurred is x will be given by the successive terms of the following series², provided that n does not appreciably alter during the course of the reaction³ :—

$$x \left\{ \left(\frac{N-1}{N} \right)^n + n \left(\frac{N-1}{N} \right)^{n-1} \left(\frac{1}{N} \right) + \frac{n(n-1)}{1 \cdot 2} \left(\frac{N-1}{N} \right)^{n-2} \left(\frac{1}{N} \right)^2 + \dots + \frac{n(n-1) \dots (n-r+1)}{r!} \left(\frac{N-1}{N} \right)^{n-r} \left(\frac{1}{N} \right)^r + \dots + \left(\frac{1}{N} \right)^n \right\}.$$

Let us suppose that the death of any given one of the bacteria occurs when r units of change have taken place within it, that is, that the "susceptibility" of every individual of the culture is the same. Then the number of bacteria which will have died at time t after the exposure began will be the sum of the least $n-r+1$ terms of the above series, hence we have :—

$$y = x \left\{ \frac{n(n-1) \dots (n-r+1)}{r!} \left(\frac{N-1}{N} \right)^{n-r} \left(\frac{1}{N} \right)^r + \dots + \left(\frac{1}{N} \right)^n \right\}.$$

If N be very large compared with unity, as it was in the experiments

¹ In other words, that the underlying change does not cease with the death of the bacteria, i.e. that the bacteria remain uniformly suspended in the solution of the disinfectant even after death. This condition was fulfilled in the experiments under consideration. If the bacteria were for any reason removed from the sphere of action of the disinfectant at death, for instance by falling out of suspension, then N at any moment would be equal to $N_1 - y$, where N_1 was the initial number of bacteria exposed to the disinfectant. In other words, unless y were very small in comparison with N_1 , N would vary with the time, and the time-relations observed by Madsen and Nyman and by Chick could not be obtained.

² Cf. G. Udny Yule (1911), *An Introduction to the Theory of Statistics*, London 1911, p. 289.

³ Provided, that is, that the quantity of disinfectant used up in killing all of the bacteria was evanescently small in comparison with the total quantity of disinfectant employed. This condition was obviously fulfilled in the experiments to which I have referred.

under consideration, then $\frac{N-1}{N}$ becomes equal to unity and the above equation may be somewhat simplified and written as follows:—

$$y = x \left(\frac{1}{N} \right)^r \left\{ \frac{n(n-1)\dots(n-r+1)}{r} + \frac{n(n-1)\dots(n-r+1)(n-r)}{r+1} \left(\frac{1}{N} \right) + \dots + \left(\frac{1}{N} \right)^{n-r} \right\}.$$

In any case, whatever be the absolute magnitude of N , the sum of the terms enclosed within the brackets will obviously be the same at all stages of the reaction, since it depends only upon the magnitudes of n , N , and r which, it has been assumed, do not alter during the progress of the reaction. Calling this sum μ , we have:—

$$y = \mu x.$$

Then if $x = f(t)$ is the relationship between x and the time of exposure:—

$$y = \mu f(t).$$

For instance, if $f(t)$ is of such a nature that:—

$$\log \frac{A}{A-x} = \kappa t,$$

i.e. if the reaction is “mono-molecular,” then, substituting from the above equations, we have:—

$$\log \frac{\frac{N}{\mu}}{\frac{N}{\mu} - \frac{y}{\mu}} = \kappa t,$$

whence:—

$$\log \frac{N}{N-y} = \kappa t,$$

which is the law of mortality which has been experimentally verified by the above-quoted observers for a uniform culture of bacteria.

It is obvious that these deductions are of a perfectly general character, and that we may employ N in the above equations to denote “total number of heliotropic organisms exposed to light,” “total number of muscle-fibres stimulated,” etc., just as we have employed it to denote “total number of bacteria exposed to disinfectant.” Similarly we may employ y to denote “number of reacting organisms,” “number of contracting fibres,” etc., and n to denote the corresponding quantitative conditions defining the *environment* to which the reacting units are exposed. In every such case y (= number of reacting individuals) will be proportional to x (= extent of underlying change in all of the individuals taken together) provided that N and n be constant throughout the change, and that the extent of the change within an

individual necessary to cause it to display the given "signal" (*i.e.* death, orientation towards a source of light, contraction, etc.) is also constant throughout the process, and very nearly the same for all of the individuals.

SUMMARY.

It is shown that provided the total number of individuals exposed to a constant environment which is inducing change within them be constant, and the number of units of change which must take place within any given individual in order to cause a given event be also constant, then the number of these events is a quantitative measure of the extent of the change in all of the individuals taken together.

From this it follows that the results of Madsen and Nyman and of Chick may legitimately be regarded as proving that the process which underlies disinfection obeys the time-relations and other characteristics of a mono-molecular chemical reaction.

FURTHER OBSERVATIONS ON NITROSO-BACTERIA.

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IN a former paper¹ I discussed the methods that I employed to cultivate the nitroso-bacterium. To this end both fluid and solid media were used.

I obtained my cultures in the first instance by inoculating small particles of garden soil into a solution advised by Winogradsky² consisting of 1 per 1000 ammonium sulphate, 1 per 1000 potassium phosphate, and 1 per 100 magnesium carbonate. The nitroso-bacteria developed in 70 % of the tubes and required as a rule two months to nitrify. After carrying on a strongly nitrifying culture through six successive ammonia solutions the culture appeared to be almost pure, from microscopical examination.

Continuing to use ammonia solutions I tried the effect of the presence of small quantities of organic matter in them³. The organic matter tried was introduced as either beef broth, urea, or peptone, commencing in quantities so small as 1 per 11,000. I used stronger solutions as nitrification developed, and in the case of beef broth was able to obtain nitrification when as much as 10 per 100 was present in an ammonia solution. Neither peptone nor urea was so favourable, although nitrification occurred in presence of small quantities.

I also employed solid media. Those used were silica jelly, as advised by Winogradsky, agar, and gelatine. Cultures used for inoculation were obtained from the strongly nitrifying ammonia solutions above

¹ Fremlin. *Journal of Hygiene*, III. 364-379, 1903.

² Winogradsky. *Ann. de l'Inst. Past.* IV. 1890, and V. 1891; also *Arch. des Sci. Biol. de St Pétersb.* I. 1892.

³ A. Stutzer. *Centralblt. f. Bakt.* Abt. 2, Bd. VII. 1901.

mentioned. Silica jelly with nutrient salts was a good medium, as also was ammonia agar (made from 1 per 1000 ammonium sulphate, 1 per 1000 potassium phosphate, and 1 per 100 magnesium carbonate and $1\frac{1}{2}$ % agar). I was able to obtain individual colonies both from silica and ammonia agar plates; these colonies inoculated into ammonia solutions in three tubes set up nitrification. When nitrification occurred in this solution I subcultured into bouillon agar plates and found that large numbers of colonies developed. Both bouillon agar and gelatine were also used as media. Cultures of the nitroso-bacteria from nitrified inorganic media were inoculated into these and plates poured; these plates developed a pure culture of a bacillus similar to the nitroso-bacterium. Pieces of the plates were inoculated into ammonia solutions. This was done 53 times, and of these inoculated tubes, 20 or 37 % showed nitrification. Again 19 pieces were taken from the bouillon agar plates where no colonies were observed and inoculated into ammonia solutions; in none of these did nitrification occur.

From all these results I came to the conclusion that the nitroso-bacterium can grow on ordinary media.

Since the publication of my former paper further evidence of nitroso-bacteria developing in organic matter has not been wanting. The following are results obtained by some of the workers on this subject.

(1) Coleman¹ came to the conclusion that peptone and urea in 0.5 % solution were inimical to nitrification.

(2) Niklewski² found that nitrite bacteria cannot develop in even dilute urine but are found and can develop well in manure. He says further that the nitrite bacterium is the same as that isolated from soil by Winogradsky and Omeliansky; but that these observers failed to grow it in organic matter, either from inoculating the organism in too small quantities, or from the unsuitability of their cultures.

(3) Stevens and Withers³, in a large series of experiments, found that organic matter in large amount in soils is not inimical to nitrifying bacteria.

(4) Temple⁴ obtained the most active nitrification in soils by adding cultures of nitrifying bacteria grown in manure.

(5) Fischer⁵ used blood mixed with soil to assist nitrification.

¹ Coleman. *Centralblt. f. Bakt. Abt. 2*, Bd. xx. 484.

² Niklewski. *Ibid. Abt. 2*, Bd. xxvi. 388.

³ Stevens and Withers. *Ibid. Abt. 2*, Bd. xxvii. 169.

⁴ Temple. *Ibid. Abt. 2*, Bd. xxxiv. 204.

⁵ Fischer. *Landw. Jahrbuch*. Bd. xli. 1911.

Reagents used for testing.

The tests for ammonia, nitrites and nitrates were made by a drop of fluid or a small piece of culture to one drop of the reagent on a white glazed plaque.

For ammonia—Nessler's test.

For nitrites—(1) Potassium iodide and starch solution with acetic acid; (2) sulphanilic acid and naphthylamine; (3) diphenylamine in sulphuric acid.

For nitrates—Diphenylamine in sulphuric acid.

For nitrates in presence of nitrites—Boil in saturated solution of ammonia chloride, then test with diphenylamine.

The diphenylamine solution is convenient for both nitrates and nitrites; when the solution contains nitrates without nitrites the blue colour does not develop instantaneously as it does with nitrites.

The Brucine test in my hands was not satisfactory. I obtained an equal reaction with either nitrites or nitrates.

In continuing my observations I have used cultures obtained from garden soil collected in Kent in most instances. In those cases in which they were cultivated from sewage mention is made of the fact. A culture is developed in a solution of ammonium sulphate, potassium phosphate and magnesium, or calcium carbonate in tap water. The ammonium sulphate and potassium phosphate may be in a strength of 1 per 1000 or as high as 1 in 200. I do not weigh the carbonate but certainly use quite 1 in 100 and probably much more. For some years I used light carbonate of magnesia as an alkaline carbonate, but finding that calcium carbonate (chalk) was equally good and more readily obtainable, I have used it instead in most cases for the past two or three years.

In these experiments the cultures were developed in a laboratory that has a mean annual temperature of 17°C ., varying from 19.6° in the summer to 14.1° in the winter. An incubator was not used as I required a good deal of space for the cultures.

In this paper I will try to show that the nitroso-bacterium, cultivated under certain conditions, can nitrify organic matter, including some ordinary laboratory media, and also urine. I will first describe its cultivation on ammonia agar, then the results obtained by adding organic matter to this medium. Following this I will give results obtained in agar plates containing a small amount of organic matter but no ammonia, and then in bouillon agar plates. Experiments will

then be recorded with calcium sulphate blocks used as a means of obtaining a large bulk of culture, and the action of such cultures on laboratory media and urine.

Cultivation on ammonia agar.

About 20 c.c. of ammonia agar, poured and allowed to set in a deep beaker about 5 cm. in diameter, were inoculated from a culture of nitroso-bacteria in ammonia solution that I had had for about a year in a filter of sand through which ammonia solution passed.

Nitrification occurred after some weeks. On to this nitrifying culture a layer of ammonia agar was poured to a depth of about $\frac{3}{4}$ inch; this layer also nitrified, and on examination it was found that the nitroso-bacterium was present in the culture medium. As oxidation proceeded layers of ammonia agar were added until the beaker was filled; the nitroso-bacterium was found in all the layers. The culture thus filling the beaker was used from time to time to inoculate other media and was refilled as necessary with fresh medium. Since the nitroso-bacterium appeared to grow through the medium I was led to try the same method with cultures in plates; here again I found the nitroso-bacterium in the upper layers. Control experiments with *P. aureus* showed me that a few bacteria are washed up when fresh medium is poured on so that this might account for the nitroso-bacterium in upper layers of jelly. In order to avoid any washing up of bacteria I poured thick layers of ammonia agar in sterile Petri dishes, and when they had set dropped them carefully on to cultures in plates. I found then that the nitroso-bacterium grew upwards through the added agar and permeated the whole medium in the plate. Following on from this knowledge, pieces of culture were placed in the centre of ammonia agar plates, and it was found that the nitroso-bacterium grew from this centre and spread about into all parts of the plate. It was necessary only to take a piece from the margin of a plate so inoculated, after nitrification was well established, and to place it in the centre of a fresh plate to obtain a subculture. In connection with this observation it is to be noted that as soon as nitrite reaction began it diffused rapidly through the medium.

The question then arose whether the presence of nitrite was essential to the satisfactory development of the nitroso-bacterium. To settle this point a strongly nitrifying ammonia agar culture was dialysed until all the nitrite had disappeared. Pieces were then placed in the centre of six ammonia agar plates to inoculate them. In 17 days very good

nitrification had developed in four of the six plates inoculated. From this it would appear that nitrite is not essential to the growth of this species.

Ammonia agar with added organic matter used as a medium.

Cultures of the nitroso-bacterium from inorganic solutions were inoculated into ammonia agar containing differing amounts of organic matter in order to observe if any difference occurred either with regard to length of time required to develop nitrous formation or in the amount of nitrite formed. The organic matter added was that contained in bouillon gelatine or bouillon agar; 5, 10, and 20 % of one or other of the above jellies were used in the experiments.

Ammonia agar with 5 % bouillon gelatine.

This proved to be an excellent medium. Ammonia agar cultures of the nitroso-bacterium were either inoculated into the liquid medium as ordinarily obtains in pouring plates or else a plate was poured, allowed to set, and a culture in ammonia agar placed in the centre. No difference was observed in the length of time required for nitrification, two, three, or four weeks being the most common, the difference between the cultures was that the one containing organic matter in the medium showed very much more marked nitrification than the cultures in ammonia agar without this addition and was indeed more marked than I had previously obtained in any medium.

Ammonia agar with 10 % bouillon gelatine.

This also was an excellent medium, good nitrous formation developed.

The plates were inoculated in the centre. Nitrification occurred in three weeks.

Ammonia agar with 10 % bouillon agar.

Here again a very good medium was found for the growth of the nitroso-bacterium, and in those plates inoculated in the centre the growth could be seen as a thin layer spreading outward until in some parts of the plate it would reach the margin like *B. mesentericus*, but unlike this species the growth was observed to be in the depth of the plate rather than on the surface, and this was so although the plates were inoculated on the surface!

Ammonia agar with 20 % bouillon agar.

This medium was inoculated from the 10 % bouillon agar cultures; good nitrification developed in most of these plates. More time was required before nitrification developed, but it was very pronounced after it had started. At least six weeks were required before it was well established and the development was not so certain as in the plates containing the lower percentages.

Ammonia agar with 30 % bouillon agar.

Twelve plates inoculated from 20 % bouillon agar cultures. After a year one gave very good nitrite reaction.

Potassium phosphate agar with 5 % bouillon gelatine as a medium.

Since the ammonia agar combined with a small amount of organic matter was found to be a very satisfactory medium for obtaining very marked nitrification, I thought that probably the ammonia was so well oxidised because the nitroso-bacteria had some organic matter to live on. If this was the case the nitroso-bacteria did not necessarily want ammonia as a source of food supply.

I had made many attempts to obtain an enzyme from these bacteria but had not satisfied myself up to that time; now it occurred to me that a medium containing phosphate of potash and bouillon gelatine in agar would grow the nitroso-bacterium, but as no ammonia would be supplied, I could perhaps obtain an enzyme, extract it from the plate, and then test its action on ammonia in solution.

With this object 12 plates were poured of a medium containing:

1 % potassium phosphate,

1.5 % agar,

5 % bouillon gelatine.

When they were set I inoculated them in the centre with strong nitrifying ammonia agar plate cultures. The plates were paraffined up and left by accident for $9\frac{1}{2}$ months. On opening, all save three had dried up. As a matter of routine I tested for ammonia and nitrite; to my amazement, although no ammonia was found, nitrification in all was intense!

Here was evidence that the organic matter was used and directly nitrified.

I now used this as a medium and numerous plates were poured. Nitrification occurred in nearly 50 %, sometimes it was intense, at others only poor. Several plates were inoculated with a soil, 88 % of these nitrified, but subcultures from these carried on to plates of the same medium did not nitrify, evidently not proving a suitable medium for continued activity.

Ordinary bouillon agar used as a medium.

The results obtained with plates containing bouillon gelatine caused me again to attempt the cultivation of the nitrifying bacteria on ordinary bouillon agar made from beef broth, 1 % peptone, 0.5 % sodium chloride, and 1½ % agar. I was the more inclined to this since I had found that inoculation in the centre of a plate with a good quantity of culture had proved eminently successful.

In these experiments about 20 c.c. of ordinary bouillon agar were melted and thoroughly mixed in a Petri dish with an alkaline carbonate ; the jelly was then left to set. When stiff, it was ready for the inoculation. Inoculations were always made in the centre of the plate, 2 or 3 c.c. of culture being incorporated with the agar jelly by means of a sterile spatula. The plates were then left for 24 hours and afterwards carefully paraffined to check evaporation of moisture. I find it always necessary to paraffin up plate cultures as these are frequently kept for six months or even a year before they are finally discarded, since the nitrifying bacteria are often very slow to commence operations. I poured altogether 47 of these plates.

The cultures used were very carefully selected and consisted, first, of those that had already oxidised ammonia in urine and other organic media ; secondly, one that had been cultivated in dilute and undiluted urine for four years ; thirdly, those that had been cultivated in ammonia solution for varying times, no organic matter having been added.

The following gives the number in each group, with results :

29	5	16
were from cultures in various organic media.	of cultures in urines.	from cultures in ammonia solution, one for 4½ years, one for 3½ years, seven for 2 years, seven for 6 months.
Of these, 6 showed good nitrification.	Of these, 2 showed good nitrification.	Of above, 2 showed nitrification, viz. those of 4½ and 3½ years' culture.

In these 10 plates nitrification was set up in the first instance in seven weeks, but it was seven months before it developed in the ten.

The first change observed in the plates was the formation of free ammonia. This commenced within 48 hours, and a large quantity was soon formed. The nine plates inoculated with young cultures showed not only formation of ammonia but developed also a very disagreeable odour like that of a very foul cesspool; this was absent from the other plates, which only had an ammoniacal odour lasting for a time.

Calcium sulphate blocks used for cultivation.

A method of culture found very satisfactory was carried out by the use of calcium sulphate in blocks. These were prepared by moulding this material into blocks of a size to lie in a Petri dish and allow of half an inch or so of space round them. This space was filled by the culture medium into which inoculations for subculture were made. After the block was introduced the plate was sterilised, and sterile culture medium could then be poured on. A $3\frac{1}{2}$ inch Petri dish containing a block will hold about 15 c.c. of fluid. I inoculated cultures of nitroso-bacteria into these plates, and at first two months were required for complete nitrification. When this occurred the fluid was poured off and fresh added. Less time was taken after each change of the fluid until only a week was finally required. The object of these blocks was to have a substance which the nitroso-bacteria could permeate so that one could have a large amount of active culture for testing fluids which could be readily poured off and changed.

These cultures were most useful for observing the action of the nitroso-bacterium on organic media, their success being probably due to the large amount of culture as compared with the amount of medium tested, and also to the efficient aeration of such shallow dishes.

The disadvantages were (1) that the fluid medium was liable to overflow by capillary attraction round the edge, and (2) that all examinations of the culture exposed it to contamination.

These calcium sulphate blocks were used for testing the action of nitroso-bacteria on urine, milk, peptone water, peptone beef broth, and blood serum.

Urine as a medium.

In the first instance undiluted urine with an alkaline carbonate was poured into the plates containing active cultures of nitroso-bacteria in calcium sulphate blocks. This set up marked bacterial action, nitrification ceased, gas having a disagreeable odour was produced,

and a very large amount of ammonia was formed. This fluid was poured off and water added. After a few days if no nitrification had occurred and much ammonia was still present the fluid was again poured off and more water added. Nitrification commenced later on and was soon well established.

Equal quantities of urine and water were also tried, but here again much ammonia was formed and as a rule the fluid had to be poured off and water added before nitrification developed. In some cases nitrification had been checked from six weeks to four months.

Twenty of the nitroso-bacterium cultures in calcium sulphate blocks were used for these urine tests: of these, 13 cultures had been obtained direct from sewage and the other seven were either from potassium phosphate agar and gelatine plate cultures, or from strongly nitrifying solutions from soil in ammonia solution.

The urine used has been sterile, fresh unsterilised, and also very old, stale, and disagreeable non-sterile fluid. The stale was nitrified rather more rapidly than the fresh, but after two or three lots had been used in succession on the same block nitrification was not so good. A culture rendered less active in this way could be put right by pouring on a 0.5 % ammonia solution.

It was found that urine diluted with four or six parts of water was very suitable, no odour or gas formation followed. The first quantity poured on took about three weeks to nitrify at 18° C., but after that four days to a week was long enough.

When a culture was working well the ammonia increased markedly for two days, on the third day it had diminished, and by the fourth it was gone and nitrification was very good. The decanted fluid gave no reaction to Nessler in the cold, but on distilling free ammonia was found, and on adding alkaline permanganate to the remaining fluid a good quantity of albuminoid ammonia was obtained!

Experiments with undiluted urine.

Although I had found that water was necessary with calcium sulphate blocks, on speaking to Mr Colin Frye, he considered that water should not be necessary if sufficient oxygen were present. Following that advice I took cultures that were already active and had nitrified urine; these I arranged in three groups. The first group consisted of three cultures made up of chalk and calcium sulphate; these I heaped up into dome-shaped masses and made a depression in the centre of the

top of the dome. They stood in deep trays, the culture fluid being poured into the depression in sufficient quantity to soak the culture masses and overflow into the dish. These raised cultures were to allow of penetration of air. The urine was poured from one to the other of these almost daily to assist further in aeration.

The second group consisted of ten active nitrifying cultures in Woodhead flasks, each containing 50 c.c. of urine, and these were aerated by pouring urine from one to the other as the first. The third group had six active cultures in jars with loose-fitting lids, which were treated in the same way, the urine being changed from one to the other until nitrification was complete. To control these 19 I had 22 cultures in Woodhead flasks, to each of these 50 c.c. of urine were introduced and this just covered the culture; all of these were left undisturbed. After about two months nitrification had developed in all the 41 cultures, occurring not only in those especially aerated but also in their controls!

The first change was the formation of ammonia, this developed in large amount in the first few days and gradually lessened as nitrification developed! No difference in time was noted between the aerated and non-aerated cultures, but the heaped up masses cannot be fairly estimated in comparison with the others as evaporation caused necessity for adding more urine from time to time, and to prevent too great concentration water was also added; however, nitrification developed in about the same time.

Effect of ordinary laboratory media on cultures of the nitroso-bacterium in calcium sulphate blocks that had been used for nitrifying sewage and urine.

Milk.

Milk diluted with four, eight, or 16 parts of water or 1 in 200 ammonia solution was used as a culture medium for five cultures of nitroso-bacteria in calcium sulphate plates.

In the first experiments water was used with the milk, but ammonia did not develop and nitrification was checked.

The water not proving satisfactory, the ammonia solution was tried, so that ammonia would be present in good quantity.

Milk diluted with three parts ammonia solution and some magnesium carbonate was tried on two cultures: nitrification in one case was checked, the reaction being poor for five days, the ammonia was also used up and a thick pellicle covered the fluid; on addition of more

ammonia solution however good nitrification occurred in another 48 hours.

In the second culture the nitrite reaction was lost for a week and a thick pellicle covered the fluid ; all ammonia also disappeared. This fluid was poured off and fresh ammonia solution used. Nitrite again developed and was very good by the 13th day.

Milk diluted with seven parts ammonia solution was used on three nitroso-bacteria cultures. In these also nitrification was suspended, but again returned and was very good in from one to three weeks ; it was necessary to add ammonia solution to these cultures to re-develop the nitrifying action, but no milk was added again until nitrification was well established. The further addition of milk medium to these plates inhibited nitrification to a greater extent than was the case in the first instance. A culture giving a good nitrite reaction in the presence of milk in seven days required 15 on again adding the solution containing milk.

Milk diluted with 15 parts of ammonia solution did not appear to check nitrification when added for the first time, but, as before observed, the addition of a second amount to such a culture caused some days delay in the nitrifying process.

Peptone water.

This was the ordinary 1 % solution as prepared for bacteriological work. It was diluted with equal parts of 1 in 200 ammonia solution.

This medium was used for six cultures. In all of these nitrification was good on the second day and all ammonia was used up by the 5th day : this is about the time required to use up the ammonia in an inorganic solution. The further addition of the peptone water medium on pouring off the first was followed by almost complete cessation of nitrification in four of the six plates, with formation of bubbles in five of them, and a disagreeable odour was also noted in three.

In from nine to 18 days however nitrification was re-established in all, and in only one instance was it necessary to pour off the peptone medium and add ammonia solution to obtain this result.

Peptone beef broth.

This was tried on five nitroso-bacterium cultures in calcium sulphate.

A 10 % solution in water was tried twice but nitrification was not good.

A medium was then used consisting of one part beef broth and one part 1 in 200 ammonia solution with some magnesium carbonate. This was poured on five cultures. Nitrification was checked for four or five days, and bubbles were frequently noted; in one instance a faint disagreeable odour was noticed, however by the eighth day nitrification was very good. One nitroso-bacterium culture was subjected to the above beef broth medium for two months and during that time the fluid was nitrified, poured off, and a fresh amount added six times. At the end of this time the culture continued to be exceedingly good, in fact a 50 % beef broth in ammonia solution was well suited to the cultures if used now and then.

Blood serum.

This, diluted, was used on nine cultures of nitroso-bacteria in calcium sulphate blocks. The dilution was usually one part of serum to seven. Four of the cultures were treated with serum diluted with water, the other five had serum with 1 in 200 ammonia solution poured on them. Those treated with serum and water lost the nitrification, the fluid becoming foul and numerous bubbles forming; the addition of ammonia solution re-established nitrification in from nine days to four weeks, and the nitrites were then formed in good amount.

The five plates on which the serum diluted with ammonia were poured varied: in three, very good nitrification occurred on the fifth day, in the other two nitrification ceased for a few days but was very good again after 10 and 19 days respectively.

Calcium sulphate blocks.

Cultures in Anaerobic conditions.

Eleven calcium sulphate blocks containing cultures of nitroso-bacteria were placed in these conditions by enclosing the Petri dishes with their contained culture blocks in jars used for anaerobic culture methods.

The oxygen was extracted by means of pyrogalllic acid in caustic potash solution.

For the experiment six of the cultures were surrounded with 10 c.c. of a 0.5 % solution of ammonium sulphate and potassium phosphate to which some magnesium carbonate was added. The other five cultures were surrounded with 10 c.c. of urine diluted with water to 1 in 4 to which magnesium carbonate was also added. These cultures were controlled by aerobic cultures with corresponding media.

RESULTS.

Cultures with ammonium sulphate.

No nitrites found after 14 days. Large quantity of ammonia present, but some ammonia taken up in first week.

The control cultures had formed nitrites and completely oxidised the ammonia some days before the anaerobic cultures were finally tested.

One part urine and three parts water.

No nitrites formed.

In first two or three days a good quantity of free ammonia formed, and this was present until the end of the experiment, which lasted for some days after the controls had completely oxidised the ammonia.

All above plates, on being removed from anaerobic jars and placed in a cupboard exposed to ordinary atmospheric conditions, rapidly oxidised the ammonia in their surrounding solutions.

These results confirm those made with the cultures in test tubes, the results of which were given in my last paper.

SUMMARY.

Interesting results were obtained with cultures in ammonia agar, from them it is evident that the nitroso-bacterium grows readily through this medium whether inoculated on the centre of a plate or when covered with the agar jelly itself. Further, the presence of 5 to 10 % of beef broth in the ammonia agar allows of better development.

Potassium phosphate agar containing bouillon gelatine but no free ammonia, on being inoculated with cultures of nitroso-bacteria, also developed nitrification; the organic matter was broken down, ammonia formed, and nitrites built up.

Nitrification in ordinary bouillon agar plates shows that organic matter does not necessarily form a barrier to the process; and further, that two of the cultures had only been fed with inorganic salts for three and four years respectively prior to the inoculation into the plates, is good evidence that gradual additions of organic matter are not absolutely necessary.

Calcium sulphate blocks allowed me to work with a larger amount of very active culture: from these it was found that urine was a very good culture medium indeed. Also that peptone water diluted with

water, or peptone beef broth, milk, or blood serum diluted with ammonium sulphate could be used as culture media; sometimes these checked nitrification for a time but it afterwards became well established.

The experiments with undiluted urine were wholly successful and stand as a further proof of nitrification in presence of organic matter.

From these results it is clear that the nitroso-bacterium is a very powerful nitrite-forming micro-organism, that it is not readily destroyed, and that a certain amount of organic matter is essential to its greatest development, but for this a large amount of culture must be used.

In conclusion I have to offer my deepest thanks to Sir William Power for the great help that he has given me and for the kindness that he has always shown; also to others who have most kindly advised me my grateful thanks are due.

AGGLUTINATION EXPERIMENTS WITH TYPHOID BACILLI ISOLATED FROM THE BODY.

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It is well known that typhoid bacilli, freshly isolated from enteric fever patients, vary in their capacity to undergo agglutination. The following series of experiments was carried out with the object of discovering whether these differences in agglutinability are dependent on the stage of the disease at which the bacillus is isolated, or on the body substance from which it is obtained.

The bacilli were grown from patients suffering from enteric fever, chiefly from blood, faeces, and urine, but also from vesicular rose-spots, and (post-mortem) from the spleen, bile, and mesenteric glands. They were tested by such fermentative and other methods as were considered sufficient to establish their identity as typhoid bacilli. An agglutinating serum was obtained by the inoculation of a rabbit with the stock typhoid bacillus in use in the hospital laboratory. For purposes of comparison, the bacilli were tested also with the sera of rabbits immunized in the same way against four strains of paratyphoid organisms.

In most instances in which a bacillus was isolated, it was tested also with the patient's serum, and the result compared with the results of agglutination by the stock antisera.

Finally, the bacilli were examined according to the method described by Michaelis (1911) of agglutination by means of acid solutions of varying strengths.

The bacilli were isolated from blood by adding blood withdrawn from a vein in the arm to bouillon (250 c.c.), or ox-bile (10 c.c.), and

incubating. The best results were obtained when 5 c.c. of blood was added to 10 c.c. of bile. Cultures from faeces were made according to the method described by Kendall and Day (1911). The isolating medium was a modified Endo medium, which contained 1.5% of agar instead of Endo's 4%, and which was made just alkaline to litmus instead of strongly alkaline. A platinum loopful of faeces was mixed in 10 c.c. of bouillon at incubator temperature, the tube was incubated for an hour, and three loopfuls of this bouillon were then spread on a modified Endo plate by means of a right-angled glass rod. The plate was incubated overnight. The number of colonies obtained on a plate by this means varied considerably, but 30-40 was common. The cultures from urine were made in the same way as those from faeces. From vesicular rose-spots, spleen, gall-bladder, and mesenteric glands, the cultures were made directly on agar, and were always found to be pure.

The organisms were examined after isolation, and the following tests were considered sufficient to establish their identity as typhoid bacilli:

- the production of acid without gas in glucose, maltose, and mannite;

- the production of slight permanent acidity, without clotting, in litmus milk;

- non-fermentation of lactose and saccharose;

- non-production of indol in peptone water after 7 days' growth;

- non-liquefaction of gelatin;

- colourless growth on potato;

- presence of motility.

The agglutination reactions were carried out by the microscopic method. The blood was collected in the usual way from the ear in capillary tubes, and the serum obtained was diluted with saline solution by a measuring pipette to 1-12½, 1-25, 1-50, 1-100... the series being continued until a dilution was found at which no agglutination occurred. The highest dilution in which slight but distinct agglutination occurred was reckoned as the limiting dilution. The bacilli were used in an 18-24 hours' bouillon culture. The use of this instead of an emulsion of a culture on agar slopes had always given reliable results with the laboratory organism.

Agglutinating sera were obtained by inoculating rabbits with a typhoid strain and four paratyphoid strains. Injections were given intraperitoneally at intervals of 10 days—1 c.c., 2 c.c., and 4 c.c. of a killed

24 hours' bouillon culture being introduced in successive inoculations. The animals were killed 10 days after the 3rd inoculation.

The following bacilli were used for immunization of the rabbits :

(1) *B. typhosus*, a stock strain used for about five years in the City of Glasgow Fever Hospitals for Widal reactions, and obtained originally from the spleen of a patient who died of enteric fever. This had proved a trustworthy organism.

(2) *B. paratyphosus* A (Brion-Kayser), which I received along with (3) and (5) from Dr R. M. Buchanan, Public Health Laboratory, Glasgow. These three strains had been obtained from Kral some time previously.

(3) *B. paratyphosus* A (Schottmüller).

(4) *B. paratyphosus* B (Schottmüller), obtained from Leeds, and brought originally from Vienna by Professor Grünbaum.

(5) *B. paratyphosus* B (Achard).

It was shown by experiment that growth of the laboratory bacillus in bile, and also on the modified Endo medium, did not alter its capacity to undergo agglutination.

Forty-six strains of *B. typhosus* were isolated and tested :

From blood	17
„ faeces	14
„ urine	8
„ vesicular rose-spot	1
„ spleen	4
„ gall-bladder	1
„ mesenteric gland	1
Total			46

These 46 bacilli were isolated from 42 patients. In three instances more than one bacillus was grown. In the first, a bacillus was obtained from blood and from urine, in the second from a rose-spot and from faeces, and in the third from the spleen, from the gall-bladder, and from a mesenteric gland.

In the study of the results obtained, attention was paid not only to the day of the disease on which a bacillus was isolated, and to its source, but also to the length of time which elapsed between its isolation and the day on which the tests were made. Certain observers have reported variations in agglutinability dependent on this circumstance, and bacilli found to be non-agglutinable, or only slightly agglutinable on isolation, have sometimes become completely agglutinable after standing for two or three months.

Agglutination by anti-typhoid serum.

The limit of agglutination of the stock bacillus with its own antiserum occurred at 1 : 80,000.

The 46 bacilli from patients were divided into three classes:

(1) Those agglutinated approximately as well as the stock typhoid bacillus (limiting dilution 1 : 80,000–1 : 45,000).

(2) Those in which there was moderate agglutination (limiting dilution 1 : 25,000–1 : 1600).

(3) Those in which agglutination was slight (limiting dilution 1 : 100–1 : 25), or absent at 1 : 25.

Class 1 includes 22 strains.

"	2	"	17	"
"	3	"	7	"

Of the 22 bacilli in Class 1, 5 were agglutinated quite as well as the stock typhoid bacillus. One of these was grown from a vesicular rose-spot, 1 from blood, 1 from the spleen, and 2 from faeces. Two bacilli showed no agglutination at 1 : 25—one from blood, and the other from the spleen.

Class 3 includes 1 bacillus isolated in the 1st week.

In the 2nd week, 17 bacilli were isolated:

Class 1 includes 11

"	2	"	4
"	3	"	2

In the 3rd week, 19 bacilli were isolated:

Class 1 includes 9

"	2	"	8
"	3	"	2

In the 4th week, 6 bacilli were isolated:

Class 1 includes 2

"	2	"	3
"	3	"	1

In the 5th week and after, 3 bacilli were isolated:

Class 1 includes 0

"	2	"	2
"	3	"	1

There was thus a distinct tendency for the earlier isolated bacilli to be agglutinated better than those obtained later. The earliest bacillus, however, grown on the 3rd day, was not agglutinable.

From the *blood*, 17 bacilli were grown :

Class 1 includes	6
„ 2 „	7
„ 3 „	4

From the *faeces*, 14 bacilli were grown :

Class 1 includes	11
„ 2 „	3
„ 3 „	0

From the *urine*, 8 bacilli were grown :

Class 1 includes	2
„ 2 „	5
„ 3 „	1

Class 1 includes also 2 bacilli from the spleen, and the bacillus from a rose-spot.

Class 2 includes the bacillus from the gall-bladder, and the bacillus from a mesenteric gland.

Class 3 includes 2 bacilli from the spleen.

A striking difference was thus evident between the agglutinability of the bacilli from faeces, and those from blood and urine, the bacilli from faeces being agglutinated much better than those from the two other sources.

The resemblance in respect of agglutinability between the bacilli from blood and those from urine suggests that the former come from the blood into the urine (which is the accepted view), and do not pass directly from the intestine to the bladder, as has been suggested by Blumer (1895).

It is worthy of note that in the case where three strains were obtained from one individual after death, the bacillus from a mesenteric gland was agglutinated to 1:25,000, that from the gall-bladder to 1:15,000, and that from the spleen not at all.

The length of time which elapsed between the isolation and testing of the bacilli appeared to have a slight influence on those obtained from the blood. The average number of days in this period for the 17 bacilli from blood was :

Agglutination of B. typhosus

in Class 1 (6 bacilli) 139 days

„ „ 2 (7 „) 120 „

„ „ 3 (4 „) 79 „

In the case of the 14 bacilli from faeces the average number of days was :

in Class 1 (11 bacilli) 61 days

„ „ 2 (3 „) 70 „

The bacilli in the other groups were too few to give comparable results.

The average time between the isolation and the examination of the bacilli from the blood was 117 days, and of the bacilli from the faeces 63 days. If then agglutinability developed with the lapse of time, the difference in the agglutination reactions of these two groups of bacilli must originally have been even greater, for the bacilli from the faeces, which were more agglutinable, had been kept a shorter time.

The average age of all the bacilli in Class 1 was 88 days, of those in Class 2, 93 days, and of those in Class 3, 72 days.

Agglutination by anti-paratyphoid A (Brion-Kayser) serum.

This serum agglutinated its own bacillus in a limiting dilution of 1:3000.

The stock typhoid bacillus was not affected at 1:25.

The bacilli isolated from patients were divided into three classes :

- (1) Those with the agglutination limit between 1:100 and 1:50 (1 bacillus).
- (2) Those with the agglutination limit between 1:40 and 1:25 (14 bacilli).
- (3) Those showing no agglutination at 1:25 (31 bacilli).

Week of isol	Limit of agglutination			Total
	1:100-1:50	1:40-1:25	No aggl.	
1st	0	1	0	1
2nd	1	7	9	17
3rd	0	5	14	19
4th	0	1	5	6
5th and after	0	0	3	3
Source of bac.				
Blood	0	3	14	17
Faeces	1	8	5	14
Urine	0	1	7	8

Class 2 includes also bacilli from spleen (1) and mesenteric gland (1).

Class 3 includes also bacilli from spleen (3), rose-spot (1), and gall-bladder (1).

There was a tendency for the earlier isolated bacilli to be better agglutinated.

As with the anti-typhoid serum, the bacilli from faeces were better agglutinated than those from blood or from urine, and the bacilli from urine again resembled those from blood rather than those from faeces.

The results were independent of the age of the bacilli. The average age of the bacilli in Class 1 was 33 days, of those in Class 2, 93 days, and of those in Class 3, 100 days.

Agglutination by anti-paratyphoid A (Schottmüller) serum.

This serum agglutinated its own bacillus in a limiting dilution of 1:200,000.

The stock typhoid bacillus was agglutinated to 1:60.

The 46 bacilli were divided into three classes:

- (1) Those with the agglutination limit between 1:350 and 1:200 (6 bacilli).
- (2) Those with the agglutination limit between 1:190 and 1:50 (28 bacilli).
- (3) Those with the agglutination limit between 1:40 and 1:25, and those showing no agglutination at 1:25 (12 bacilli).

Week of isol.	Limit of agglutination			Total
	1:350—1:200	1:190—1:50	1:40 and less	
1st	0	1	0	1
2nd	3	10	4	17
3rd	2	13	4	19
4th	1	2	3	6
5th and after	0	2	1	3
Source of bac.				
Blood	0	9	8	17
Faeces	3	10	1	14
Urine	1	4	3	8

Class 1 includes also bacilli from gall-bladder (1), and mesenteric gland (1).

Class 2 includes also bacilli from spleen (4), and rose-spot (1).

The degree of agglutination was independent of the time of isolation of the bacillus.

With this serum also the bacilli from faeces were agglutinated considerably better than those from blood.

The time between the isolation and testing of the bacilli in Class 1 was 82 days, of those in Class 2, 97 days, and of those in Class 3, 133 days. Agglutination seemed to be diminished rather than increased with increase in the age of the organism.

Agglutination by anti-paratyphoid B (Schottmüller) serum.

This serum was the most active obtained and agglutinated its own bacillus to 1:800,000.

The stock typhoid bacillus was agglutinated to 1:150.

The bacilli were divided into three classes:

- (1) Those with the agglutination limit between 1:400 and 1:200 (8 bacilli).
- (2) Those with the agglutination limit between 1:190 and 1:50 (32 bacilli).
- (3) Those with the agglutination limit between 1:40 and 1:25, and those not agglutinated at 1:25 (6 bacilli).

Week of isol.	Limit of agglutination			Total
	1:400—1:200	1:190—1:50	1:40 and less	
First 3 weeks	8	26	3	37
4th week and after	0	6	3	9
Source of bac.				
Blood	2	12	3	17
Faeces	3	10	1	14
Urine	1	5	2	8

Class 1 includes also bacilli from spleen (1), and mesenteric gland (1).

Class 2 includes also bacilli from spleen (3), rose-spot (1), and gall-bladder (1).

The bacilli obtained in the first three weeks were agglutinated better than those grown later.

The bacilli from faeces were agglutinated in somewhat higher dilutions than those from blood, but the difference was not so marked as with the preceding sera.

The average age of the bacilli in Class 1 was 91 days, of those in Class 2, 111 days, and of those in Class 3, 138 days. Agglutinability thus did not increase with age.

Agglutination by anti-paratyphoid B (Achard) serum.

This serum agglutinated its own bacillus in a limiting dilution of 1:70,000.

The stock typhoid bacillus was agglutinated to 1:400.

The bacilli were divided into three classes :

- (1) Those with the agglutination limit between 1:400 and 1:200 (11 bacilli).
- (2) Those with the agglutination limit between 1:190 and 1:50 (28 bacilli).
- (3) Those with the agglutination limit between 1:40 and 1:25 (7 bacilli).

Week of isol.	Limit of agglutination			Total
	1:400—1:200	1:190—1:50	1:40—1:25	
First 3 weeks	10	23	4	37
4th week and after	1	5	3	9
Source of bac.				
Blood	4	9	4	17
Faeces	4	9	1	14
Urine	1	5	2	8

Class 1 includes also bacilli from rose-spot (1), and mesenteric gland (1).

Class 2 includes also bacilli from spleen (4), and gall-bladder (1).

The bacilli isolated in the first three weeks were agglutinated somewhat better than those obtained later.

The bacilli from faeces were agglutinated a little better than those from blood, but the difference was too slight to justify any conclusion.

The average age of the bacilli in Class 1 was 96 days, of those in Class 2, 84 days, and of those in Class 3, 122 days. Agglutinability did not increase with the age of the organisms.

*General conclusions from the results of the experiments
with antisera.*

- (1) The bacilli which were isolated earlier in the disease tended to be agglutinated better by artificial antisera than those isolated later, the difference being most marked between those obtained in the first three weeks, and those grown after the end of that time.

(2) The bacilli isolated from faeces were agglutinated much better by anti-typhoid serum, and somewhat better by anti-paratyphoid serum than those grown from the blood.

(3) The bacilli isolated from urine resembled those grown from blood rather than those from faeces.

(4) The length of time which elapsed between the isolation of the bacilli and their examination exercised no appreciable influence on their power to undergo agglutination.

Agglutination reactions of bacilli with the serum of the patient from whom each was isolated.

The results of the agglutination reactions carried out with the patients' sera and the respective bacilli come now to be considered.

Thirty-seven bacilli were investigated in this way :

From blood	13
.. faeces	14
.. urine	8
.. rose-spot	1
.. spleen	1

In this series of experiments no fixed standard of comparison in respect of a limiting dilution was available, as the sera varied in activity. The results obtained were therefore compared quantitatively with the results of the agglutination tests with the same sera and the stock typhoid bacillus. In a few instances, it was found that the patient's bacillus was agglutinated rather better than the stock bacillus by his own serum, but the difference was so slight that I found it convenient to regard the result of agglutination with the stock bacillus as the maximum in each case.

In these cases the tests were carried out within a few days of the isolation of the bacilli.

The 37 bacilli were divided into three classes :

Class 1 includes those which were agglutinated approximately as well as the stock typhoid bacillus.

Class 2 those which were agglutinated distinctly less well than the stock typhoid bacillus.

Class 3 those which were agglutinated much less well than the stock typhoid bacillus, or were not agglutinated.

In Class 1 the ratio of the agglutination limit of the autogenous bacilli to that of the stock bacillus was from a little above 1 to $\frac{1}{2}$.

In Class 2 the ratio was from $\frac{1}{2}$ to $\frac{1}{6}$.

In Class 3 the ratio was below $\frac{1}{16}$.

Class 1 includes 26 bacilli

"	2	"	8	"
"	3	"	3	"

Class 1 includes 1 bacillus isolated in the 1st week.

Of 17 bacilli isolated in the 2nd week

Class 1 includes 13

"	2	"	4
"	3	"	0

Of 13 bacilli isolated in the 3rd week

Class 1 includes 10

"	2	"	1
"	3	"	2

Of 6 bacilli isolated in the 4th week and after

Class 1 includes 2

"	2	"	3
"	3	"	1

It is evident that the capacity of the bacilli to undergo agglutination was independent of the time of isolation.

Of 13 bacilli isolated from *blood*

Class 1 includes 12

"	2	"	1
"	3	"	0

Of 14 bacilli isolated from *faeces*

Class 1 includes 8

"	2	"	5
"	3	"	1

Of 8 bacilli isolated from *urine*

Class 1 includes 4

"	2	"	2
"	3	"	2

Class 1 includes also the bacilli from rose-spot and spleen.

The bacilli from blood were very much better agglutinated than those from either faeces or urine.

General conclusions from results of experiments with patients' sera.

(1) With the patient's serum no connection could be made out between the time of isolation of a bacillus and the degree of agglutination present.

(2) The bacilli from blood were agglutinated very well indeed, and very much better than those from faeces.

Results of agglutination tests with the patients' sera and the artificial serum compared.

The 37 bacilli tested with their respective patient's serum were included in the 46 tested with the artificial sera. A comparison of the results of agglutination tests on the bacilli with the two kinds of serum, the patient's and the anti-typhoid, shows striking differences.

With regard to the time of isolation, as has been seen, the anti-typhoid serum agglutinated bacilli isolated in the earlier stages of the disease much better than those obtained later; whereas with the patients' sera no such difference was found to exist. No connection could be made out in the latter instance between the time of isolation of the bacillus and the degree of agglutination present.

A consideration of the bacilli from the point of view of their origin showed the following results. (The classes were arranged as before.)

Class 1 includes bacilli agglutinated approximately as well as the stock typhoid bacillus.

Class 2 includes bacilli agglutinated distinctly less well than the stock bacillus.

Class 3 includes bacilli agglutinated much less well than the stock bacillus, or not at all.

Of 13 bacilli isolated from *blood*

	With the anti-typhoid serum	With the patient's serum
Class 1 includes	3	12
„ 2 „	6	1
„ 3 „	4	0

Of 14 bacilli isolated from *faeces*

	With the anti-typhoid serum	With the patient's serum
Class 1 includes	11	8
„ 2 „	3	5
„ 3 „	0	1

Of 8 bacilli isolated from *urine*

	With the anti-typhoid serum	With the patient's serum
Class 1 includes	2	4
„ 2 „	5	2
„ 3 „	1	2

Class 1 includes also bacilli from rose-spot and spleen.

While with the anti-typhoid serum the bacilli from blood were less well agglutinated than those from faeces, with the sera of the respective patients they were agglutinated very well indeed, and very much better than those from faeces. The latter responded less well, indeed, to the agglutinative action of the autogenous serum than to that of the anti-typhoid serum. Here again the bacilli grown from urine resembled those obtained from blood rather than those from faeces, agglutination with the patient's serum being decidedly better than with the anti-typhoid serum.

*Summary of comparison of results with anti-typhoid serum
and with patients' sera.*

(1) With the anti-typhoid serum the bacilli isolated earlier in the disease were agglutinated much better than those obtained later; with the patients' sera, no such difference was found.

(2) With the anti-typhoid serum the bacilli from faeces were agglutinated much better than those from blood; with the patients' sera, the bacilli from blood were agglutinated very much better than those from faeces. These results were obtained with the same strains of bacilli.

(3) The bacilli from faeces were agglutinated rather less well by the patients' sera than by the anti-typhoid serum.

Discussion of variations in agglutinability.

It is known that under certain circumstances bacilli which normally are well agglutinated by an appropriate serum may become non-agglutinable (Paltauf, 1912). One of these circumstances is the passage of the bacillus through the body of man or an animal. According to Porges and Prantschoff (1906) "lessened agglutinability is chiefly observed in cultures freshly isolated from the body, or passed through animals: in bacilli from exudates: and in bacilli which have been passed through media containing agglutinins."

The cause of this phenomenon has been variously regarded. Porges (1905) showed that typhoid bacilli which had been rendered completely non-agglutinable by heating to 80° C. had their agglutinability restored by washing in normal saline solution. He supposed that the nuclein split off the nucleo-protein of the organisms was the substance which inhibited agglutination, and that when this was removed by washing, the bacilli again became agglutinable. The capsulated bacteria, such as Friedländer's bacillus, are normally non-agglutinable, and Porges and Prantschoff (1906) attributed this to increased formation of protein. In four non-agglutinable strains of typhoid bacilli, isolated from spleens, these observers thought they could detect the presence of a capsule.

Another suggestion (Paltauf) is that certain strains of typhoid bacilli are really composite strains containing both agglutinable and non-agglutinable members. Under certain circumstances, the latter may come to predominate.

Culture in agglutinin-containing media brings about non-agglutinability. Sacquépée (1901) caused strains of typhoid bacilli to become less agglutinable by growing them in collodion sacs in the peritoneal cavity of rats immunized against *B. typhosus*. The change, however, took place slowly, and it was only after treatment of a series of subcultures in the same way that the agglutinability was reduced, at the end of five months, to $\frac{1}{6}$ of its original standard. He concluded that non-agglutinable strains in man were produced by the growth of the organism in an infected or immunized body.

Numerous observers have recorded the isolation of typhoid bacilli which were agglutinated only slightly or not at all (Horton Smith (1900), Remy (1901), Sacquépée (1901), Cambier (1902), Emery (1902), Nicolle and Trenel (1902)). These bacilli have fulfilled all the tests for typhoid bacilli, and in certain cases it has been found that immunization of animals against feebly agglutinable strains resulted in the production of a serum which agglutinated laboratory strains.

According to Paltauf, not infrequently agglutination is lessened with the patient's serum as well as with an artificial anti-typhoid serum.

A slow rise on the part of these bacilli to a normal standard of agglutinability has been described by most authors as taking place, either after a certain number of subcultures, or simply by the lapse of time, without subculture. (Cambier, Emery, Porges and Prantschoff, Lipschütz (1904).) Porges and Prantschoff found that four non-agglutin-

able strains from spleens were agglutinated as well as stock bacilli after about 15 subcultures on agar, and Lipschütz noted a similar rise in the case of three typhoid strains isolated from urine. In the latter instance, these three strains were not definitely agglutinated in a dilution of 1 : 200 by an active serum (agglutinating to 1 : 20,000), whereas, three months later, without subculturing, they were agglutinated by the serum to 1 : 20,000.

A change in the characters of an organism by its presence in an animal body is referred to by Besredka (1909), in a criticism of work done by Aronson (1902, 1903) on streptococci. Aronson had endeavoured to prove the identity of streptococci from various sources by means of experiments on animals. He immunized horses with a streptococcus which he had rendered extremely virulent by passage through a series of mice, and found that streptococci from other sources, also rendered virulent by passage through mice, were acted on by the serum of these horses equally with the original strain used for immunization. From this he concluded that all the streptococci tested were essentially the same. Besredka criticised Aronson's conclusions on the ground that all his streptococci had been modified by their passage through mice, and that each strain had become what he called "*un streptocoque de passage*."

Conclusions from personal observations.

From what has been said it is plain that when typhoid bacilli circulate in the blood they sometimes undergo a change which manifests itself in diminished agglutinability. The results of my experiments seem to show that the bacilli in the faeces are less changed from the original agglutinable type than those in the blood, which are acted on to a much greater extent by the body fluids. But this explanation, that the bacilli become non-agglutinable by growth in the body of a person whose blood contains immune substances, does not account for the fact that the bacilli isolated from blood were practically all agglutinated by the serum in which they were circulating as well as was the stock typhoid bacillus. It may be that after the organisms have been modified by the action of the serum, some alteration in the serum itself is called forth by the change in the bacilli, and this might account for the fact that the bacilli grown from faeces were agglutinated rather worse by their respective patient's serum than by the artificial serum.

As has been shown, the bacilli isolated earlier in the disease were agglutinated better than those obtained later, and this, so far as it goes, is in favour of Saequépée's theory that non-agglutinability is produced by growth in a body containing immune substances. It is to be noted, however, that a bacillus isolated from the blood on the 3rd day of illness was unacted on by the anti-typhoid serum at 1:25. On the day on which this bacillus was obtained, the patient's serum caused no clumping of the stock bacillus at 1:25. The non-agglutination of this bacillus, therefore, must have been due to some cause other than growth in an agglutinin-containing medium.

Agglutination of bacilli by acid solutions (Michaelis).

Another method was employed in the attempt to differentiate the bacilli which had been already tested by antisera.

Michaelis (1911) pointed out that many strains of bacteria are agglutinated by acids, and that a fixed degree of acidity corresponds to the maximum of agglutination. This maximum, he says, is characteristic for individual strains of bacteria, and can be used as a help in their identification.

The test is carried through as follows:

The bacillus to be examined is grown on agar slopes for 24 hours and is then emulsified in distilled water, the emulsion being rather denser than that used for a Widal reaction. The following six solutions are required:

	Normal sodium hydrate	Normal acetic acid	Distilled water
1	5 c.c.	7.5 c.c.	87.5 c.c.
2	5	10	85
3	5	15	80
4	5	25	70
5	5	45	50
6	5	85	10

1 c.c. of each of these solutions is put into each of a series of six test-tubes, and to each tube is added 3 c.c. of the bacterial emulsion. The tubes are then shaken up and put in the incubator at 37° C. When the first agglutination appears, the row of tubes is taken from the incubator and left at room temperature for some time. In any case the tubes are not kept at 37° C. for more than an hour.

According to Michaelis, with typhoid bacilli agglutination occurs only in tubes 3, 4, and 5, as a general rule. It is commonly most

marked in tube 3, though occasionally in 4, and in 2 and 5 is much slighter, if it occurs in these at all. Tube 3 is therefore reckoned as the optimum for *B. typhosus*.

B. paratyphosus has its optimum in tubes 5 and 6, but the A and B strains cannot be distinguished from one another.

B. coli is usually not agglutinated.

Rost (1911) applied the test to eight strains of *B. typhosus*, a paratyphoid A strain, a paratyphoid B strain, and other organisms. The results he obtained with typhoid bacilli agreed with those of Michaelis; with the paratyphoid B there was marked agglutination in tube 6, and with the paratyphoid A no agglutination. He concluded that the method is "a valuable addition to our resources for diagnosing typhoid."

A later investigator, Jaffé (1912), criticised the method. He tested 41 strains of *B. coli*, 40 of *B. typhosus*, 11 of *B. paratyphosus* A, three of *B. paratyphosus* B, and three of *B. typhi murium*, with unsatisfactory results. Eleven of the *B. coli* strains showed agglutination, and the test gave no assistance in the differentiation of the atypical members of the *B. coli* group. With *B. typhosus* the results were no more certain. In the 40 strains the optimum occurred in tubes 2 and 3. In 22 agglutination was present only in 1 or 2 tubes, in five from tube 2 to tube 6, and in one in all 6. (It was found that this last bacillus was agglutinated by distilled water.) In the case of four of the bacilli no agglutination occurred. Of the 11 paratyphoid B strains, two showed agglutination in tubes 4 and 5, eight in tubes 4, 5, and 6, and one from tube 3 to tube 6. The optimum varied. Of the three paratyphoid A strains, two were agglutinated in tubes 4, 5, and 6, and one in tubes 3 to 6. Here also the optimum varied.

This method was applied to the 46 bacilli tested by means of the antisera, with the exception of one from blood, which had died out: to two stock typhoid strains, the laboratory bacillus, and the R.A.M.C. typhoid strain (Rawlings)¹; to the four stock paratyphoid strains; and to a strain of *B. coli*.

By accident, a bacillus was tested twice on different days, and this was discovered only when the results were tabulated. The results obtained were precisely the same on each occasion. This points to a constancy of the results obtained by the method.

With regard first to the stock typhoid bacilli, both were agglutinated in tubes 3 to 6, the laboratory strain having its maximum in tube 4, and

¹ Kindly supplied to the hospital by Sir William Leishman.

B. typhosus (Rawlings) in tubes 3-5. With the four paratyphoids, the maximum occurred in each case in tube 6, agglutination in the case of Brion-Kayser A being slight, and present only in this tube, while with the three others there was some agglutination also in tube 5. *B. coli* was unaffected.

The agglutination which took place in the case of the 45 bacilli from patients varied in extent and degree, but the maximum was found to occur as follows:

In tube 1	0 times
„ 2	0 „
„ 3	23 „
„ 4	3 „
„ 5	4 „
„ 6	5 „
In tubes 4, 5 and 6...	1 „
„ 5 and 6	1 „
No agglutination	8 „
Total			45 „

That is to say, in half the cases the maximum of agglutination occurred in tube 3, which Michaelis regarded as typical for *B. typhosus*.

The place of occurrence of the maximum was independent of the time of isolation, and the only difference among the bacilli from the point of view of their origin was that the bacilli from urine seemed to be less "typical" in reaction than those from blood or faeces.

With regard to the number of tubes showing a reaction in each case, agglutination was found more frequently in the combination of tubes 3 to 5 than in any other. In four instances irregular agglutination was present but in each of these the maximum occurred in tube 3. In nine cases agglutination was of Michaelis' "paratyphoid type." In four of the latter agglutination was present only in tubes 5 and 6. In one case in which it was found in tubes 4-6, and in four others with agglutination in tubes 3-6, the maximum occurred in tube 5 or tube 6.

The bacilli showing the "paratyphoid reaction" with acids were not better agglutinated than the others by the anti-paratyphoid sera.

From these results it seems that the test is of interest rather than value in the examination of typhoid bacilli.

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THE RESISTANCE OF THE VACCINE VIRUS TO FILTRATION.

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WHETHER the vaccine virus is filterable or not has been the subject of experiment and discussion for many years and since Negri's results published in 1905, from which he deduced that the virus was filterable, numerous experiments dealing with the subject have been made by many workers.

The consensus of opinion appears to be that the vaccine virus is capable of passage through a filter such as a Berkefeld V, and although a few experimenters have failed to obtain positive results it is usual to include the vaccine organism in the list of filter passers. It is so included by Löffler (1913) and Lipschütz (1913), and Paschen (1911) states that the evidence for the filterability of the lymph organism is overwhelming.

The main difficulty throughout with lymph filtering experiments has been that in the absence of artificial culture of the virus, one has had to deal with the ordinary lymph "emulsion," a thick conglomeration of vesicular material, in which the specific organism is embedded. In attempting to use a filter to negotiate this slimy mass the chief difficulty, and a very important one, has been that, after the first few seconds of filtering under the necessary pressure, the bougie has been covered externally with a slimy coat which transforms the character of, say, a Berkefeld V candle to that of a much finer one; almost, perhaps, to the condition of a gelatine filter. The result has been that, while critics of positive filtration results have felt suspicious of the filters

being intact, critics of negative filtration results have pointed to the fact that a slime-coated bougie is calculated to prevent the passage through it of any kind of micro-organism.

In order to overcome this difficulty the following technique was suggested to me by Professor C. J. Martin. The vesicles of calves, 96 hours after vaccination, were clamped, and portions of their clear fluid contents rapidly transferred, by means of a graduated Pasteur pipette, to a measured quantity of a solution of sodium citrate and distilled water. This expressed fluid consists microscopically of white blood cells and a few staphylococci. The presence of the vaccine virus in an active condition is demonstrated by the vaccination of a suitable animal with the fluid. By using this material for filtration not only was the slimy mass of epithelial cells in various stages of dissolution avoided, but clotting of the expressed lymph was obviated, and a clear fluid was obtained for experiment, which filtered with ease under moderate pressure, passing through the filter almost as readily as water.

0.25 c.c. vesicular exudate was admixed with 2 c.c. of 5 % sodium citrate solution. This small quantity can be dealt with easily for filtration if a sufficiently small filter be used. The smallest pattern Berkefeld V laboratory filter was obtained, and its metal mount and a portion of the adjacent part of the bougie were removed, leaving about 3 cm. of the distal part of the bougie intact. The hollow of the bougie was carefully reamed out so as to make it circular at the orifice and into this prepared hollow surface was fitted one end of a piece of circular glass tubing, which had been covered with thin rubber tubing. A perfect fitting joint was thus obtained, and a small filter prepared capable of introduction into an ordinary test-tube and of dealing adequately with the small quantity of fluid available for the experiment.

Using this technique, the lymph of nine calves have been experimented with. Six separate samples were taken from each calf, making a total of 54 experiments.

The filtrates were inoculated on guinea-pigs, as also were portions of the unfiltered fluids to act as controls. Subsequently the inoculations were repeated on calves, being stored at 4° C. until opportunity for calf-inoculation arose. In these experiments, in which the filtration of calf vaccine has been reduced in simplicity to the filtration of water, in no single instance has any vesiculation or any visible trace of specific reaction resulted from the inoculation of any of the filtrates, while in every instance, without exception, the controls have set up definite and typical vesiculation.

Criticism may possibly be directed against the use of guinea-pigs as tests for calf vaccine activity. If guinea-pigs are vaccinated on the back or abdomen they are not very satisfactory as test animals. But if only buck pigs are used and these are not already immune and are vaccinated with active virus on the scrotum, typical and usually exceptionally fine vesicles result. The advantages of using these small animals are that one animal can be used exclusively for each experiment—a proceeding not usually possible with calves—and the tests can be carried out in a place isolated from general vaccination work, thus eliminating the chance of contamination with other sources of vaccination. These guinea-pig tests are in themselves so satisfactory that corroboration by calf experiments is scarcely necessary, but as mentioned above, the inoculations were repeated on calves. As was pointed out by more than one speaker at the morning session of the Bacteriological Section of the International Medical Congress in London, on August 11th, 1913, no positive results can be fully accepted when the tests have been carried out where routine work connected with the test virus is in progress; and still less is such a test to be relied on when made on animals, other areas of whose skin are inoculated with a virus of a like nature to the test virus.

To account for the failure on the part of some observers to filter the vaccine virus, Negri has suggested that the virus of vaccine lymph which has been stored will pass through a filter when the virus of recent lymph would not pass. Negri bases this view on the assumption that small filterable forms of the virus occur as the result of two or three weeks' storage. I therefore made a further series of 36 experiments with lymph prepared in the way described above, which had been stored for some weeks. Portions of the expressed lymph of six calves were pipetted into citrate solutions as before, but the lymph dilution in this series was 1 in 13. 36 samples from the six calves were then taken. These were stored at 4° C. for three weeks with the addition of 1 c.c. of pure glycerine to each sample of citrated lymph. At the end of three weeks' storage, the unfiltered control portions produced good vesiculation on guinea-pigs and upon calves, but in no instance was any trace of reaction seen after vaccination with the filtrates.

From the foregoing 90 experiments I am forced to conclude:

(1) That the specific virus of vaccinia, as contained in the clear lymph obtained from vesicles 96 hours after vaccination of the calf, is either of such a size that it is unable to pass through large filter pores such as a Berkefeld V filter possesses, or

(2) It is contained within some body which is of such a size that it is incapable of such passage, or

(3) It loses its virulence by passage through a Berkefeld V filter, which seems improbable.

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AN INQUIRY INTO THE RELATION BETWEEN SOCIAL STATUS AND CANCER MORTALITY.

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CONSIDERABLE difference of opinion has existed as to whether the incidence of cancer is at all affected by the habits of life of different classes of the community. There is of course no doubt that in certain occupations particular forms of malignant disease are exceptionally prevalent, the case of chimney sweeps being notorious; but the more general question as to the incidence on large groups of occupations has not been so definitely determined. The present research was suggested by the apparently contradictory results obtained by Heron from his study of London statistics, and by Maynard from an analysis of United States data.

We have, we think, obtained a satisfactory explanation of this contradiction, the conclusion in this case being sufficiently clear; on the other hand, the exact interpretation of the statistical constants derived from other material is by no means so definite as we could wish. Nevertheless our observations seem to us likely to prove of interest to other students of the problem. The paper is divided into the following sections:

(1) We consider the method employed and results obtained by Heron.

(2) We analyse the data given in the last decennial supplement issued by the Registrar General in so far as they bear upon this problem.

(3) We deal with some direct measurements based upon income and wages statistics.

In the paper published by Maynard in 1910, he remarked that in studying the association between cancer and occupations arranged

in the presumed order of social status the coefficients calculated appeared to show that status and cancer death-rates were negatively correlated, the occupations of highest status having lowest death-rates. Age corrections were employed and the coefficients of correlation were calculated by the method of ranks, and by that of four-fold division.

Since the appreciation of social status is to some extent a matter of personal opinion more than one classification was used, but re-arrangement in this way did not very sensibly affect the correlations, which were of the order of $-.4$ to $-.6$. The source of the material was the Registration Area of the United States. Maynard specifically called attention to the contradiction between his results and those of Heron, to which we now turn.

The conclusions at which Heron arrived are contained in his famous memoir entitled, "On the Relation of Fertility in Man to Social Status and on the changes in this relation that have taken place during the last fifty years."

His remarks on the present topic were merely incidental and our criticisms do not have any bearing upon his main theme. In the first place Heron found the correlation between the birth-rate (based on the proportion of legitimate births per 100 married women, aged 15 to 54) and the general death-rate from cancer per 100,000 persons, to be $-.563 \pm .089$. This finding, he remarked, seemed to require further investigation.

The following series of coefficients was then obtained :

Variables						Correlation
Female cancer rate and birth-rate						$-.535 \pm .093$
Male	"	"	"	$-.156 \pm .127$
Female	"	"	proportion of domestic servants			$+.404 \pm .109$
Male	"	"	"	"	"	$+.422 \pm .107$
Female	"	"	"	"	professional men	$+.553 \pm .088$
Male	"	"	"	"	"	$+.447 \pm .104$

He then remarks, "These results seem to indicate that the conditions of prosperity and culture which lead to a low birth-rate also conduce to a high cancer death-rate. In other words, cancer cannot, like phthisis, be taken as a measure of that unhealthy environment with which a high birth-rate seems to be associated."

Further on he also states that "Cancer alone of the undesirable physical conditions dealt with so far seems more prevalent in the prosperous and cultured districts, and to be associated with a lower birth-rate."

These results seemed at first sight very definite and some were tempted to explain them by the supposition that the diagnosis would be likely to be more accurate in the better class districts.

It appears to us, however, that no such explanation need be invoked, but that in fact Heron's coefficients do not measure what he seems to have supposed them to measure. In the data used by him the cancer rates are the ordinary crude values, viz. deaths per 100,000 persons, males and females respectively.

Since the age distribution varies considerably in the different boroughs, and since the incidence of cancer is greatly influenced by the age constitution of the population, the coefficients shown may merely mean that in the poor class districts the age constitution is unfavourable to the occurrence of cancer. We have tested this matter closely and the results will probably convince the reader of the justice of our criticism. To begin with, we determined the correlation between the crude cancer death-rate (*persons*) used by Heron, and the proportion of professional men as stated in his paper for the year 1901, and then the correlation after the cancer rates had been corrected for age and sex distribution, using the figures published by the Medical Officer for the County of London. We omitted the Boroughs of Deptford and Greenwich in both calculations, because they were taken together by Heron, but shown separately in the Medical Officer of Health's tabulation of corrected rates. The values are $+ \cdot 284 \pm \cdot 122$ in the case of corrected rates, and $+ \cdot 711 \pm \cdot 065$ employing crude rates, the former being only doubtfully significant.

We then calculated the correlation between both the crude and corrected cancer death-rates (*persons*) for 1906-10 and the proportion of domestic servants per 100 private families as shown in the recent census. The correlation when the corrected cancer death-rates are used is $- \cdot 032 \pm \cdot 127$, but when the crude cancer rates are employed the coefficient rises to $+ \cdot 562 \pm \cdot 087$.

If we use as a measure of poverty the proportion of persons over seventy years of age, in receipt of old age pensions on 31st March, 1911, and take once more the corrected cancer rate (*persons*) 1906-10 (Table I), the correlation is $+ \cdot 200 \pm \cdot 122$. In other words, we reach no significant association between measures of high or low social status in the London Boroughs and corrected cancer death-rates.

As further evidence of the difference between the results in accordance with whether crude or corrected rates be employed, we tabulate below some values yielded in the case of cancer and fertility (the latter

TABLE I.

Showing proportion of persons over 70 years of age in receipt of Old Age Pensions on the 31st of March, 1911, and the corrected cancer death-rate 1906-10 for 28 Metropolitan Boroughs.

	Number of pensioners per 1000 persons over 70 years of age (March 31st, 1911)	Corrected cancer death- rate (persons) 1906-10		Number of pensioners per 1000 persons over 70 years of age (March 31st, 1911)	Corrected cancer death- rate (persons) 1906-10
Chelsea	408	1.02	Hackney	502	.94
Fulham	475	1.08	Stoke Newington	445	1.07
Hammersmith	539	1.05	Poplar	595	.93
Kensington	310	.96	Stepney	480	.98
Westminster	397	1.01	Deptford	551	.99
Hampstead	272	.97	Greenwich	446	.91
Paddington	403	1.06	Woolwich	528	1.02
St Marylebone	399	1.12	Camberwell	547	.97
Finsbury	681	.96	Lewisham	392	.94
Holborn	448	.99	Bermondsey	729	1.11
St Pancras	492	1.09	Southwark	619	1.02
Islington	529	1.06	Lambeth	527	1.08
Bethnal Green	633	1.04	Battersea	569	1.06
Shoreditch	503	.93	Wandsworth	380	.95

factor again being based upon the proportion of legitimate births to married women, aged 15 to 54, in the case of the 1901 figures; for the later figures the birth-rate is based upon married women, 15-45, as shown in the census of 1911), and a series of other correlation coefficients designed to test the matter thoroughly, attention being paid to sex.

In view of these results we consider that Heron's inferences respecting cancer are mistaken and that, consequently, there is no necessary contradiction between Maynard's coefficients for the United States and the real condition of affairs in London.

We then attempted to investigate the relation between social class and cancer death-rate by means of the Registrar General's Decennial Analysis of occupational mortality. It will be remembered that the Decennial Supplement provides comparative mortality figures for a large number of different occupations—the data being available for "Occupied Males," 1890-2; for "Occupied Males," 1900-2, and for "Occupied and Retired Males," 1900-2. The plan we proposed to ourselves was the following:

The occupations, having upwards of 20,000 males engaged therein¹, were to be divided into a series of groups, supposed to correspond roughly

¹ This limit was adopted in order to give some steadiness to the rates.

TABLE II.

Variables	Correlations	
	Corrected cancer death-rate	Crude cancer death-rate
26 Metropolitan Boroughs (1901):		
Birth-rate (married women 15-54) and cancer death-rate (<i>Persons</i>)... ..	$-.002 \pm .132$	$-.555 \pm .092$
28 Metropolitan Boroughs (1901):		
Birth-rate (married women 15-54) and cancer death-rate (<i>Females</i>)	$-.177 \pm .126$	$-.535 \pm .093$ (Heron)
Do. do. do. (<i>Males</i>)	$.242 \pm .122$	$-.156 \pm .127$,,
28 Metropolitan Boroughs (1906-10):		
Birth-rate (married women 15-45) and cancer death-rate (<i>Persons</i>)	$-.102 \pm .126$	$-.608 \pm .080$
28 Metropolitan Boroughs (1911):		
Birth-rate (married women 15-45) and cancer death-rate (<i>Persons</i>)... ..	$-.122 \pm .126$	$-.677 \pm .069$
Do. do. do. (<i>Females</i>)	$-.315 \pm .115$	$-.701 \pm .065$
Do. do. do. (<i>Males</i>)	$.118 \pm .126$	$-.421 \pm .105$
26 Metropolitan Boroughs (1901):		
Proportion of professional men per 1000 occupied males and cancer death-rate (<i>Persons</i>)...	$.284 \pm .122$	$.711 \pm .065$
28 Metropolitan Boroughs (1901):		
Proportion of professional men per 1000 occupied males and cancer death-rate (<i>Females</i>)	$.283 \pm .119$	$.553 \pm .088$ (Heron)
Do. do. do. (<i>Males</i>)	$.049 \pm .130$	$.447 \pm .104$,,
28 Metropolitan Boroughs (1906-10):		
Proportion of persons per 1000 over 70 in receipt of Old Age Pensions on Mar. 31, 1911 and cancer death-rate (<i>Persons</i>)	$.200 \pm .122$	$-.409 \pm .106$
28 Metropolitan Boroughs (1911):		
Proportion of persons per 1000 over 70 in receipt of Old Age Pensions on Mar. 31, 1911 and cancer death-rate (<i>Persons</i>)	$.213 \pm .122$	$-.473 \pm .099$
Do. Females do. do. (<i>Females</i>)	$-.040 \pm .127$	$-.398 \pm .107$
Do. Males do. do. (<i>Males</i>)	$.243 \pm .120$	$-.300 \pm .116$
28 Metropolitan Boroughs (1901):		
Proportion of domestic servants per 100 private families and cancer death-rate (<i>Females</i>) ...	$.093 \pm .129$	$.382 \pm .111$
Do. do. do. (<i>Males</i>)	$.020 \pm .130$	$.454 \pm .103$
Proportion of domestic servants per 100 females and cancer death-rate (<i>Females</i>) ...	$.080 \pm .129$	$.400 \pm .109$
Do. do. do. (<i>Males</i>)	$-.003 \pm .130$	$.451 \pm .109$
28 Metropolitan Boroughs (1906-10):		
Proportion of domestic servants per 100 private families and cancer death-rate (<i>Persons</i>) ...	$-.032 \pm .127$	$.562 \pm .087$

to social rank. The occupational rates were then to be distributed in these groups and from the arrays so formed the correlation ratio was to be determined. The difficulties to be faced were somewhat numerous. We shall set them out in order.

(1) Classification is necessarily somewhat a matter of opinion. Thus, our original classes were: (a) Professional, (b) Clerical and Commercial, (c) Shopkeepers and Shop Assistants, (d) Skilled workers, (e) Domestic Servants, (f) Unskilled workers. Although it would probably be admitted by most people that these groups do roughly correspond to the social strata of the nation, much difference of opinion might arise as to the absolute propriety of the divisions, *e.g.* domestic work would, by some, be placed, not in a class of its own, but with the skilled trades, or by others again with the unskilled trades, and, even if the classes were accepted, there must again be differences of opinion as to the one to which particular occupations ought to be assigned, *i.e.* whether certain trades are "skilled" or "unskilled." We have allowed for these objections in two ways. Firstly, the number of classes has been reduced and the correlation ratio re-calculated. Secondly, the whole list of occupations has been separately classified by four independent observers, the classifications, which differed in three cases, being used for the re-determination of the constants.

(2) The question also arises as to whether the rates should be weighted with the number of workers in each trade or profession, and opinions as to the propriety of such weighting might differ. We have calculated the constants twice in each case, *i.e.* with and without weighting. The small number of occupations in certain groups, *e.g.* domestic workers, is compensated for to some extent by the process of weighting.

(3) The exact interpretation of the correlation ratio in such a case as the present, in view of the difficulties of grouping, is certainly not so distinct as that of the ordinary coefficient of correlation (r). As, however, we could not calculate the product moment r , owing to the qualitative nature of our classification, the next best method seemed to be the use of the correlation ratio, notwithstanding the objection which we freely admit might be taken to this course.

(4) It has been impossible to get an accurate distribution of trades into the different classes, owing to the fact that the classification of occupations adopted by the Registrar General for the purpose of calculating the comparative mortalities includes, in the same industry, persons of widely different status; *e.g.* in the builder's group there are

comprised builders, bricklayers, builders' labourers and bricklayers' labourers. In classifying this group, most observers would say that the building industry is a skilled trade, but when the large proportion of unskilled men is taken into account, it is clear that some modification ought to be made and that any conclusions arrived at from such a grouping must be adopted with extreme caution. For this reason we are inclined to attach less importance to the method of weighting in this particular instance than we might otherwise have done.

In the following tables the results of this somewhat laborious analysis are set out:

TABLE III*.

Association between occupation and cancer mortality.
1st Classification.

(a) *Occupational mortality unweighted with the number of males engaged in each trade.*

	Mean cancer death-rate	S.D.	Coeff. of variation	6 Groups		5 Groups		4 Groups	
				η	Corrected η^\dagger	η	Corrected η^\dagger	η	Corrected η^\dagger
1890-2									
"Occupied Males"	55.00 \pm .74	9.72	17.66	.28 \pm .07	.12 \pm .08	.28 \pm .07	.17 \pm .07	.24 \pm .07	.15 \pm .07
1900-2									
"Occupied Males"	64.91 \pm 1.03	13.79	21.24	.43 \pm .06	.36 \pm .06	.42 \pm .06	.36 \pm .06	.42 \pm .06	.37 \pm .06
1900-2									
"Occupied and Re- tired Males"	68.57 \pm 1.12	15.07	21.98	.47 \pm .06	.41 \pm .06	.45 \pm .06	.40 \pm .06	.44 \pm .06	.40 \pm .06

(b) *Occupational mortality weighted with the number of males engaged in each trade.*

	Mean cancer death-rate	S.D.	Coeff. of variation	6 Groups η^\ddagger	5 Groups η^\ddagger	4 Groups η^\ddagger
1890-2						
"Occupied Males"	52.63 \pm .68	8.88	16.88	.20 \pm .07	.19 \pm .07	.19 \pm .07
1900-2						
"Occupied Males"	64.09 \pm 1.20	16.07	25.07	.37 \pm .06	.36 \pm .06	.35 \pm .07
1900-2						
"Occupied and Re- tired Males"	67.71 \pm 1.30	17.41	25.71	.40 \pm .06	.38 \pm .06	.38 \pm .06

* The "probable errors" in this and subsequent tables have been computed from the formula $.67449 \times \frac{1 - \eta^2}{\sqrt{n}}$ where n is the number of occupations used. This formula is not strictly appropriate, but perhaps sufficient as a rough and ready test of reliability.

† See Pearson, *Biom.* viii. 254-6. We used the formula $\eta^2 = \frac{\bar{\eta}^2 \cdot (\kappa - 1)/N}{1 - (\kappa - 2)/N}$, where $\bar{\eta}$ is the observed value and κ the number of arrays.

‡ Corrected values of η are not given for the weighted groups, as if N be taken as the sum of the weights, no appreciable difference is made by correction.

TABLE IV.

*Association between occupation and cancer mortality.**2nd Classification.**(a) Occupational mortality unweighted with the number of males engaged in each trade.*

	Mean cancer death-rates	S.D.	Coeff. of variation	6 Groups		5 Groups		4 Groups	
				η	Corrected η^*	η	Corrected η^*	η	Corrected η^*
00-2									
Occupied Males "	55.00 \pm .74	9.72	17.66	.22 \pm .07	?†	.18 \pm .07	?†	.12 \pm .08	?†
00-2									
Occupied Males "	64.57 \pm 1.01	13.52	20.95	.23 \pm .07	?†	.21 \pm .07	?†	.21 \pm .07	.08 \pm .07
00-2									
Occupied and Re-tired Males "	68.15 \pm 1.10	14.68	21.54	.27 \pm .07	.10 \pm .07	.25 \pm .07	.11 \pm .07	.24 \pm .07	.14 \pm .07

(b) Occupational mortality weighted with the number of males engaged in each trade.

	Mean cancer death-rates	S.D.	Coeff. of variation	6 Groups η^*	5 Groups η^*	4 Groups η^*
1890-2						
" Occupied Males "	52.61 \pm .68	8.88	16.89	.28 \pm .07	.15 \pm .07	.15 \pm .07
1900-2						
" Occupied Males "	64.09 \pm 1.15	15.36	23.96	.26 \pm .07	.26 \pm .07	.25 \pm .07
1900-2						
" Occupied and Re-tired Males "	67.49 \pm 1.28	17.13	25.37	.30 \pm .07	.30 \pm .07	.29 \pm .07

* See Pearson, *Biom.* VIII, 254-6.† Corrected η is not determinable in these instances η being $< \sqrt{\frac{\kappa-1}{N}}$.

‡ See Footnote † to Table III.

TABLE V.

*Association between occupation and cancer mortality.**3rd Classification.**(a) Occupational mortality unweighted with the number of males engaged in each trade.*

	Mean cancer death-rates	S.D.	Coeff. of variation	6 Groups		5 Groups		4 Groups	
				η	Corrected η^*	η	Corrected η^*	η	Corrected η^*
00-2									
Occupied Males "	55.00 \pm .74	9.72	17.66	.24 \pm .07	?†	.24 \pm .07	.08 \pm .08	.24 \pm .07	.14 \pm .07
00-2									
Occupied Males "	64.57 \pm 1.01	13.52	20.95	.30 \pm .07	.17 \pm .07	.30 \pm .07	.20 \pm .07	.29 \pm .07	.22 \pm .07
00-2									
Occupied and Re-tired Males "	68.22 \pm 1.10	14.71	21.56	.33 \pm .07	.22 \pm .07	.32 \pm .07	.23 \pm .07	.31 \pm .07	.25 \pm .07

* See Pearson, *Biom.* VIII, 254-6.† Corrected η is not determinable in these instances η being $< \sqrt{\frac{\kappa-1}{N}}$.

(b) Occupational mortality weighted with the number of males engaged in each trade.

	Mean cancer death-rates	S. D.	Coeff. of variation	6 Groups η^s	5 Groups η^s	4 Groups η^s
1890-2						
" Occupied Males "	52.61 \pm .68	8.89	16.89	.17 \pm .07	.16 \pm .07	.11 \pm .08
1900-2						
" Occupied Males "	64.09 \pm 1.15	15.36	23.96	.26 \pm .07	.26 \pm .07	.25 \pm .08
1900-2						
" Occupied and Re- tired Males "	67.49 \pm 1.28	17.13	25.37	.30 \pm .07	.30 \pm .07	.29 \pm .07

* See Footnote † to Table III.

It will be seen that the values of η reached vary very considerably, according to which of the different classifications is taken. We have given the corrected value of η in the case of the unweighted observations wherever this was determinable but it will be noticed that in several instances this could not be done, η being less than $\sqrt{\frac{\kappa-1}{N}}$ ¹. In the case of the weighted observations, however, corrected values have not been given, as no appreciable difference would be made if N were taken to be the sum of the weights.

The highest values are given for " Occupied and Retired " (1900-2), these ranging from .41 to .10 in the unweighted, and from .40 to .29 in the weighted observations, using the corrected ratio in the former case. For the " Occupied only " group in the same period, the ratios range from .36 to zero and from .37 to .26 respectively. By re-grouping into five or four groups the values are reduced somewhat in the case of weighted observations, but are of substantially the same order.

The results for the earlier period under investigation (1890-2) are considerably smaller than those for 1900-2, but in view of the fact that the returns for 1890-2 are rather less reliable and not strictly comparable with those of the later period, owing to alterations in the Registrar General's classification, we do not consider too much importance should be attached to the earlier figures.

A general review makes it appear, however, that the association as measured by η can hardly be zero and a study of the following tables (VI, VII, and VIII), which show mean cancer rates, weighted and unweighted, within the different groups, indicates that the sign of the association is negative, that is to say, the cancer death-rate tends to diminish with increasing social status.

¹ See Pearson, *Biom.* VIII. 256.

TABLE VI.

Constants calculated for the arrays of occupation (6 Groups).

1st Classification.

(a) *Unweighted with number of males engaged in each occupation.*

	1890-2 "Occupied Males"				1900-2 "Occupied Males"				1900-2 "Occupied & Retired Males"			
	Mean cancer death-rate	S.D.	Coeff. of variation	No. of occupations in array	Mean cancer death-rate	S.D.	Coeff. of variation	No. of occupations in array	Mean cancer death-rate	S.D.	Coeff. of variation	No. of occupations in array
Professional men	51.25	7.94	15.49	6	61.67	10.17	16.50	6	64.17	11.79	18.37	6
Clerks & Commercial Men	58.75	13.86	23.60	4	65.50	6.78	10.35	5	69.50	4.00	5.76	5
Shopkeepers & Assistants	53.46	9.41	17.60	13	58.65	8.47	14.44	13	62.12	10.46	16.84	13
Skilled Workers	53.88	8.14	15.11	40	62.80	10.49	16.70	41	65.85	10.99	16.69	41
Domestic Servants	58.75	10.83	18.48	2	81.67	12.05	14.75	3	90.83	10.27	11.31	3
Unskilled Workers	59.81	10.31	17.23	13	74.46	20.67	27.76	14	79.29	22.29	28.11	14

(b) *Weighted with number of males engaged in each occupation.*

	1890-2 "Occupied Males"				1900-2 "Occupied Males"				1900-2 "Occupied & Retired Males"			
	Mean cancer death-rate	S.D.	Coeff. of variation	No. of occupations in array	Mean cancer death-rate	S.D.	Coeff. of variation	No. of occupations in array	Mean cancer death-rate	S.D.	Coeff. of variation	No. of occupations in array
Professional Men	49.56	7.19	14.50	182	57.37	10.68	18.61	187	60.09	11.58	19.27	187
Clerks & Commercial Men	51.42	10.11	19.66	514	65.87	5.31	8.06	523	70.89	3.38	4.77	571
Shopkeepers & Assistants	53.63	11.20	20.89	704	59.12	7.55	12.78	721	62.33	9.43	15.13	721
Skilled Workers	51.44	7.19	13.97	4605.5	60.49	9.09	15.03	4788	63.29	9.21	14.55	4797.5
Domestic Servants	58.43	15.13	25.89	105	82.24	12.24	14.88	181	91.75	10.69	11.65	181
Unskilled Workers	54.95	9.36	17.04	2280.5	72.44	25.62	35.37	2142	76.98	27.62	35.88	2193.5

TABLE VII.

2nd Classification.

(a) *Unweighted with number of males engaged in each occupation.*

	1890-2 "Occupied Males"				1900-2 "Occupied Males"				1900-2 "Occupied & Retired Males"			
	Mean cancer death-rate	S.D.	Coeff. of variation	No. of occupations in array	Mean cancer death-rate	S.D.	Coeff. of variation	No. of occupations in array	Mean cancer death-rate	S.D.	Coeff. of variation	No. of occupations in array
Professional Men	51.25	7.94	15.49	6	61.67	10.17	16.50	6	64.17	11.79	18.37	6
Clerks & Commercial Men	58.75	13.86	23.60	4	65.50	6.78	10.35	5	69.50	4.00	5.76	5
Shopkeepers & Assistants	53.33	8.76	16.43	15	60.33	9.01	11.61	15	63.67	10.54	16.56	15
Skilled Workers	55.18	9.63	17.46	41	70.24	13.96	19.88	42	67.98	14.45	21.25	42
Domestic Servants	62.50	12.91	20.66	3	75.00	8.04	10.72	3	82.50	7.07	8.57	3
Unskilled Workers	55.28	6.71	12.14	9	68.25	18.73	27.44	10	73.00	21.67	29.69	10

(b) *Weighted with number of males engaged in each occupation.*

	Mean cancer death-rate	S.D.	Coeff. of variation	1000's of males engaged	Mean cancer death-rate	S.D.	Coeff. of variation	1000's of males engaged	Mean cancer death-rate	S.D.	Coeff. of variation	1000's of males engaged
	Mean cancer death-rate	S.D.	Coeff. of variation	1000's of males engaged	Mean cancer death-rate	S.D.	Coeff. of variation	1000's of males engaged	Mean cancer death-rate	S.D.	Coeff. of variation	1000's of males engaged
Professional Men	49.57	7.17	14.46	183	57.35	10.68	18.62	183	60.00	11.58	19.27	187
Clerks & Commercial Men	51.43	10.11	19.66	513.5	65.99	5.07	7.68	562	70.89	3.38	4.77	571
Shopkeepers & Assistants	51.53	10.35	19.36	825.5	60.78	8.29	13.65	818.5	63.90	9.50	14.87	846
Skilled Workers	51.83	7.93	15.31	4823	62.89	10.87	17.28	4849.5	64.39	11.50	17.85	4936
Domestic Servants	64.52	9.65	14.96	320	73.34	6.61	9.01	320	79.70	5.30	6.65	321
Unskilled Workers	52.82	8.58	16.24	1711	70.08	26.02	37.14	1763	75.24	29.31	38.95	1790

TABLE VIII.

3rd Classification.

(a) *Unweighted with number of males engaged in each occupation.*

		1890-2 "Occupied Males "					1900-2 "Occupied Males "					1900-2 "Occupied & Retired Males "				
		Mean cancer death-rate	S.D.	Coeff. of variation	No. of occupations in array	Mean cancer death-rate	S.D.	Coeff. of variation	No. of occupations in array	Mean cancer death-rate	S.D.	Coeff. of variation	No. of occupations in array			
Professional Men	...	54.29	10.46	19.26	7	62.50	9.64	15.42	7	65.36	10.88	16.65	7			
Clerks & Commercial Men	...	54.17	13.12	24.23	3	65.00	7.50	11.54	4	68.75	4.15	6.03	4			
Shopkeepers & Assistants	...	53.39	9.07	16.98	14	59.46	8.70	14.62	14	62.86	10.43	16.59	14			
Skilled Workers	...	54.09	8.37	15.47	41	63.93	11.91	18.68	42	67.26	12.37	18.38	42			
Domestic Servants	...	60.00	15.21	25.34	2	76.25	11.39	14.94	2	85.00	7.50	8.82	2			
Unskilled Workers	...	60.23	10.31	17.11	11	71.88	21.62	30.08	12	76.46	23.50	30.73	12			

(b) *Weighted with number of males engaged in each occupation.*

		Mean cancer death-rate	S.D.	Coeff. of variation	1000's of males engaged	Mean cancer death-rate	S.D.	Coeff. of variation	1000's of males engaged	Mean cancer death-rate	S.D.	Coeff. of variation	S.D.	Coeff. of variation	1000's of males engaged
Professional Men	...	55.51	11.79	21.24	247	59.98	10.21	17.02	247	63.29	11.35	17.94	11.35	17.94	252
Clerks & Commercial Men	...	48.43	6.67	13.78	449.5	65.80	5.35	8.14	498	70.08	3.54	5.00	3.54	5.00	506
Shopkeepers & Assistants	...	53.49	10.49	19.62	803	60.46	7.98	13.19	795.5	63.59	9.44	14.85	9.44	14.85	823
Skilled Workers	...	52.10	7.89	15.11	5005	62.01	10.27	16.56	5039.5	64.59	10.92	16.90	10.92	16.90	5124.5
Domestic Servants	...	58.43	15.13	25.89	105	75.07	11.34	15.11	105	84.15	7.45	8.86	7.45	8.86	106
Unskilled Workers	...	53.94	9.66	17.92	1760.5	70.91	26.10	36.81	1810	76.05	29.53	38.83	29.53	38.83	1839.5

In view of (1) the irregular distribution of means from class to class, (2) the alterations effected by re-grouping and differences in weighting, it is plain that conclusions must be drawn with caution. We do not feel justified in asserting more than that there appears to be some slight association between a high cancer death-rate and low occupational status. This result is in qualitative agreement with that of Maynard.

We next attempted to obtain a direct quantitative measure of status in the following way. The average earnings in a large number of occupations were recorded in the wages census of the Board of Trade 1906.

We used (1) Wages, (2) Comparative Cancer Mortality figures, 1900-2, (3) number of persons employed, and found the partial correlation between (1) and (2) for (3) constant. The value of $+0.138 \pm 0.092$ resulted. Since this value is derived from a selection, the census taking no account of professional earnings, we should *prima facie* anticipate a higher value were we able to extend the process to the whole population. This result is apparently in conflict with those described above and as the method is more direct, we should assign some importance to it, but, apart from the fact that the probable error is so large, that, for that reason alone, we can hardly base any arguments upon the value of the correlation actually obtained, another difficulty arises.

As we have pointed out elsewhere, the cancer rates for each industry are not calculated upon a basis that would lend themselves to a division into groups comparable as to wages, owing to the wide range of status within the trades. It may be therefore that in any one class of workmen, although an average wage with a corresponding cancer rate near the mean is shown, in reality the rates are weighted at one end with a large number of persons in receipt of high wages and having a low cancer rate, and at the other end with a large number of persons having a high cancer death-rate and low wages. As we are unable correctly to estimate the truth of this contention we cannot say to how far the correlation would be modified, or even in what direction, had our occupations been less selected and at the same time our cancer rates more representative of the position of affairs within each actual wage group.

Lastly, we made another direct measurement in the case of the City of Hamburg. In that city, deaths from cancer are separately tabulated for each subdivision, and the average income of the inhabitants is also recorded.

TABLE IX.

Showing deaths from cancer 1906-1912, corrected cancer death-rate and average income 1904-10 for 26 divisions of the city of Hamburg.

	Population 1910	Cancer deaths 1906-1912	Corrected cancer death-rate per 1000 per 7 years	Average income, Marks
Altstadt-Nord	20,440	228	9.7	612
Altstadt-Süd	9,141	78	7.6	840
Neustadt-Nord	40,603	349	7.6	663
Neustadt-Süd	30,877	274	8.1	475
St Georg-Nord	41,140	328	6.6	888
St Georg-Süd	61,291	426	7.7	475
St Pauli-Nord	39,954	316	7.8	560
St Pauli-Süd	35,026	255	6.9	485
Eimsbüttel	117,941	652	6.0	614
Rotherbaum	31,478	217	5.9	2,696
Harvestehude	25,233	151	5.8	3,679
Eppendorf	72,100	416	5.8	622
Winterhude	32,422	172	6.5	1,210
Barmbeck	93,241	461	5.0	417
Uhlenhorst	41,556	254	6.6	1,085
Hohenfelde	31,091	260	6.7	1,523
Eilbeck	54,907	337	5.9	830
Borgefelde	34,230	198	5.4	676
Hamm	44,624	226	5.8	756
Horn	7,826	53	7.2	503
Billwärder Ausschlag	46,945	255	6.6	364
Veddel	5,847	41	7.7	374
Means		270.3	6.77	924.9

Excluding a few very small districts, the populations of which were too small to give reasonably reliable rates, 26 were available and we had cancer for 1906-12 and income statistics for the years 1904-10. Corrected cancer death-rates were calculated (the statistics of the populations at ages in the districts, which are not published, were kindly sent us by the authorities of the Hamburg Public Health Department), and the correlation between cancer rate and average income was deduced. The value proved to be -0.306 ± 0.130 . As this coefficient is 2.4 times its probable error some significance may attach to it, but we must not forget that (1) average income in the case of a great commercial city may be but a poor measure of status, *e.g.* it is possible that the incomes assessed in any one district do not refer exclusively to residents in that district, and (2) some of the districts are not very large, so that

rates deduced even from the returns of as many as seven years may not be altogether reliable.

However, this result is probably less open to criticism than any others we have obtained and serves to strengthen the general conclusion that cancer is not more, but less fatal among the well-to-do classes.

The results of the present enquiry may be summarized as follows :

(1) Heron's conclusion that cancer in London is associated with conditions of higher social status is dependent, we think, upon an erroneous method of calculation and is not borne out when cancer rates corrected for age are employed.

(2) An analysis of occupational mortality leads to the suggestion that cancer is less fatal among the higher social or economic classes, but the results are somewhat irregular.

(3) In Hamburg, average income is negatively correlated with the rate of cancer mortality.

We cannot bring this paper to a conclusion without expressing our hearty thanks to Dr M. Greenwood, Jr., of the Lister Institute of Preventive Medicine, for assistance and advice given in the course of this investigation.

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STUDIES IN SPONTANEOUS PHAGOCYTOSIS.

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PART I.

Phagocytosis as an adsorptive process.

BOTH the sensitisation of typhoid bacilli by serum and their ingestion by leucocytes when sensitised, have been shown by Ledingham (1912) to take place in accordance with the laws regulating an adsorption process, the removal of opsonin from a serum by bacteria, and of sensitised bacteria from an emulsion by leucocytes following very satisfactorily the adsorption equation $y = kx^n$, where y = the amount adsorbed, x = the amount left free and k and n are constants.

It remained to be seen whether spontaneous phagocytosis, that is, phagocytosis occurring in the absence of serum, either active or inactive, also follows a similar law.

For this purpose a *Staphylococcus aureus* was employed, thick emulsions of an overnight agar slope culture being made in physiological saline and a series of dilutions prepared therefrom. The leucocytes used were those present in an emulsion of human blood corpuscles, taken up in citrate solution and washed three times with physiological saline.

Equal volumes of staphylococcal emulsion, leucocyte emulsion and saline were incubated in a shaker at 37° C. for half an hour, at the end of which time the mixtures were removed, films drawn, fixed and stained. In each film never less than 100 leucocytes were counted. Besides the phagocytic index thus obtained, the ratio of cocci to

leucocytes was calculated, the coccal emulsion having been counted in one of its dilutions in a Thoma-Zeiss counting chamber and the leucocytes taken as being roughly 8000 per c.mm. This approximation is allowable, seeing that for each series the leucocytic emulsion is the same throughout. Reckoning from the number of cocci that were available for each leucocyte and the number which each, on the average, had actually taken up, as given by the phagocytic index, one obtained the percentage ingested.

In the following experiments, I-VI, one notices first of all that the results obtainable in a spontaneous phagocytosis experiment are not so reliable as when serum is present. There is, for instance, only a rough consistency with regard to the index one may expect from a coccal emulsion of a certain strength, although it must at the same time be remembered that the leucocyte emulsion varied in strength from experiment to experiment to a certain extent and would introduce a variable factor.

Experiment I.

Cocci per c.mm.	Cocci Leucocytes	Phagocytic index	% of cocci ingested
$22,000 \times 10^6$	2,750	30.58	1.11
$11,000 \times 10^6$	1,375	—	—
$5,500 \times 10^6$	687.5	9.53	1.39
$2,750 \times 10^6$	343.75	1.62	0.47
$1,375 \times 10^6$	171.875	4.53	2.64
688×10^6	85.9375	4.35	5.06

Experiment II.

$11,448 \times 10^6$	1,431	15.55	1.09
$5,774 \times 10^6$	715.5	20.12	2.81
$2,862 \times 10^6$	357.75	7.68	2.15
$1,431 \times 10^6$	178.875	7.25	4.04
715×10^6	89.4375	4.45	4.97
358×10^6	44.7187	3.26	7.29

Experiment III.

$3,120 \times 10^6$	390	9.3	2.4
$1,560 \times 10^6$	195	4.1	2.1
780×10^6	97.5	3.1	3.2
390×10^6	48.75	1.26	2.6
195×10^6	24.375	1.94	7.9
97.5×10^6	12.1875	0.92	7.5
48.75×10^6	6.0937	0.29	4.8
24.375×10^6	3.0468	0.32	10.5

Experiment IV.

$7,696 \times 10^6$	962	12.2	1.3
$3,848 \times 10^6$	481	5.2	1.1
$1,924 \times 10^6$	240.5	7.2	3.0
962×10^6	120.25	2.2	1.8
481×10^6	60.125	1.1	2.3
240.5×10^6	30.0625	1.2	4.0
120.25×10^6	15.0312	1.2	8.0
60.125×10^6	7.5156	1.05	14.0
30.0625×10^6	3.7578	0.34	9.1

In Exps. V and VI the actual strength of the coccal emulsion was not estimated. Consequently the percentage of cocci ingested could not be determined, but the ratio of the relative strength of coccal emulsion to the phagocytic index gave a similar indication as to the adsorptive nature of the process.

*Experiment V.**Experiment VI.*

Strength of coccal emulsion	Phagocytic index	Phagocytic index	Phagocytic index	Phagocytic index
		Relative no. of cocci		Relative no. of cocci
32x	49.05	1.53	10.78	0.34
16x	17.60	1.10	17.61	1.10
8x	14.52	1.81	13.18	1.65
4x	9.64	2.41	12.97	3.21
2x	4.84	2.42	11.02	5.51
x	4.86	4.86	9.20	9.20

Turning to the figures representing the percentage of cocci ingested, it is to be noted that while they suggest that the process of ingestion is of an adsorptive nature, there being with a decrease in the number of cocci available, a marked rise in the percentage ingested, these figures are too variable to be used mathematically, the experimental error apparently preventing more accurate proof as had been possible in the case of phagocytosis in the presence of serum.

PART II.

The influence of hydrogen-ions on phagocytosis.

The influence of acid and alkali on spontaneous phagocytosis has been considered by Hamburger and Hekma (1908). They used *horse leucocytes* and as object for phagocytosis *carbon particles*, taking the percentage of leucocytes that had ingested any carbon at all as index. They came to the conclusion that any addition either of acid or alkali

to the phagocytic system, resulted in a lowering of the index, this being particularly the case with acid. It must be noted, however, that they worked with comparatively strong solutions of H_2SO_4 and NaOH , the strength in the mixture of leucocytes and carbon particles being from $n/20$ to $n/200$ in the case of the acid and for the alkali from $n/100$ to $n/500$.

In an endeavour to find proof for electrochemical influence on the phenomenon of phagocytosis, a series of experiments was carried out in which phagocytosis took place under the influence of concentrations of acid and alkali weaker than those used by Hamburger and Hekma and in the absence of serum either active or inactive, control experiments being performed without either of these additions and simply in the presence of physiological saline solution.

In these experiments the organism used throughout was again a *staphylococcus aureus*, emulsions of it and the leucocytes being prepared as described above.

Acetic acid and ammonia were the acid and alkali employed, both in dilutions of $n/50$ and $n/330$, physiological saline being used to prepare the dilutions in place of distilled water. One volume of the staphylococcal emulsion was added to an equal volume of either acid, alkali or saline as the case might be, the two digested at room temperature for varying periods, then a third and equal volume of leucocyte emulsion added, and the whole incubated at 37°C . for half an hour, a shaker being employed to maintain a homogeneous distribution of the leucocytes throughout the mixture. At the end of the half hour's incubation films were made, fixed and stained in the usual way. The volumes used were capillary ones, the same capillary pipette serving for the measurement of all three volumes. For each count 100 leucocytes were taken. All slides were examined under cipher, as only in this way can subjective influences on one's counting be avoided; and by re-counting slides and comparing separate counts from the same slide it was seen that the error occurring in this part of the experiment was very small. Yet it was soon apparent, as had been the case in the earlier adsorption experiments, that, in a phagocytic system not containing serum, the experimental error was greater than when phagocytosis took place in the presence of serum; a series of control experiments demonstrated that an average error of about 10 % above or below the average was to be expected, though individual counts might fall still further out of line. Therefore it could only be on larger differences in the indices that one could lay any stress, or by grouping a number of

experiments together, that it was possible to arrive at reliable conclusions.

I have been unable to explain the reason of this rather wide reach of error. The greatest care was always taken that the conditions, under which the various experiments were carried out, should be as similar as possible. It was thought that the irregularity might be due to the use of imperfectly cleansed glass ware, traces of serum still adhering to the small tubes from previous experiments, or that glass tubing had been used the walls of which gave off alkali on being drawn out into pipettes. This idea was tested by having all glass specially cleaned and by the use of Jena glass, but no improvement in the result took place.

Notwithstanding these irregular indices it was quite obvious that the tendency was for digestion of the cocci with acid to give a raised count, while digestion with alkali apparently brought about a fall, though this latter did not seem to be so pronounced.

TABLE I.

Digestion with	Phagocytic index			Saline count brought to 100		
	1'	30'	1½ hrs.	1'	30'	1½ hrs.
Acetic acid <i>n</i> /50	22.55	34.81	25.36	116	202	137
Acetic acid <i>n</i> /330	18.54	18.61	37.59	96	108	203
Physiological saline	19.37	17.20	18.52	100	100	100

In Table I is given a typical experiment in which the cocci were digested for various periods with the two different strengths of acetic acid before the addition of the leucocytes to the system. A well-marked increase in phagocytosis is noticeable in those cases where the stronger acid or a longer period of digestion was given.

TABLE II.

Digestion with	Phagocytic index				Saline count brought to 100			
	1'	30'	2½ hrs.	6½ hrs.	1'	30'	2½ hrs.	6½ hrs.
Ammonia <i>n</i> /50	10.90	9.12	11.61	15.32	82	60	64	84
Ammonia <i>n</i> /330	12.63	12.87	14.52	29.47	94	85	80	161
Physiological saline	13.37	15.19	18.04	18.29	100	100	100	100

In Table II is shown a corresponding experiment in which digestion with ammonia took place. As will be seen, the general effect is to lower the indices, but this lowering is not as marked as is the increase in the case of digestion with acid.

In both tables are appended columns in which the acid and alkali indices are stated relative to saline indices of 100 ; this with the purpose of giving a more comprehensive view of the numbers. One point has to be decided before reading the results from such experiments, especially in view of the fact that sometimes, as is seen in Table II, with an increase in the time of digestion with saline itself, a rise in the phagocytic index is observable. The point is, might not this rise possibly be due to a multiplication of the cocci during the digestion period, and the greater rise, in the case of digestion with acid, to an accelerated multiplication ? However, by counting the number of cocci in the emulsions on mixing with saline, acid and alkali, and after these mixtures had stood for varying periods at room temperature up to several hours, it was seen that no appreciable increase in the numbers of the cocci had taken place and that consequently the elevation of the phagocytic index after digestion could not be due to the simple fact of there being more cocci available at a later period of digestion than at an earlier.

A striking feature of these experiments is, that an alteration in the length of digestion, or in the strength of acid or alkali used, does not result in any regular corresponding alteration in the phagocytic index. If there should be, as there well might be, differences due to these factors, they are apparently not sufficiently marked to escape masking by the irregularities to which the counts are liable. One must have recourse to the average and to judge from this, as shown in Table IV, one would conclude that the weaker acid produces the greater increase in phagocytosis and the stronger alkali the greater decrease.

TABLE III.

Digestion with	Digestion period																			
	1'	1'	1'	15'	15'	15'	30'	30'	30'	30'	30'	30'	30'	30'	30'	1½ h.	1½ h.	2½ h.	5½ h.	6½ h.
Acetic acid n/50	116	—	—	271	113	272	119	202	135	89	202	44	163	261	137	30	—	351	—	—
Acetic acid n/330	96	—	—	240	106	818	—	108	—	153	210	34	135	179	203	29	—	195	—	—
Physiologi- cal saline	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
Ammonia n/330	94	47	106	—	106	286	85	—	150	34	—	—	58	—	—	—	80	121	161	37
Ammonia n/50	82	30	136	—	117	90	60	58	59	77	—	—	59	—	—	—	64	75	84	39

Table III is a summary of all experiments, with the indices given so as to correspond in each experiment with a saline index of 100. Arranged as the indices are, in order of length of digestion, it is clearly

seen how little the amount of rise or drop appears to depend on this factor, but that notwithstanding the aberrant indices, that fall right out of line (four of the 28 indices obtained under the influence of acid and six of the 27 indices obtained under the influence of alkali give in the former case markedly lowered, in the latter case markedly raised counts), the general tendency is for counts to be raised by the addition of acid and if anything to be lowered by the addition of alkali. This is more clearly observed by taking the average of all counts and comparing them as has been done in Table IV, where it is seen that digestion with acid produces an index considerably above the saline one, while digestion with alkali one on the whole slightly below it.

TABLE IV.

Digestion with			Average phagocytic index	
Acetic acid $n/50$	167) 179
Acetic acid $n/330$	193	
Physiological saline	100	
Ammonia $n/330$	105) 90
Ammonia $n/50$	76	

This lowering of the phagocytic index in alkali digestion however, taking the average of all experiments, does not fall beyond the limit of experimental error; it is only when the series of indices obtained under the influence of the stronger ammonia solution ($n/50$) is grouped together that a definite retarding effect on phagocytosis is observed.

The results of these phagocytosis experiments seem clearly to point to an acceleration of the spontaneous ingestion of cocci by leucocytes when under the influence of acid, while by the addition of alkali to a spontaneous phagocytic system very little difference is occasioned unless the stronger alkali be employed, in which case phagocytosis is retarded to a certain extent.

Oker-Blom (1912) working also with a *staphylococcus*, but employing ionic solutions somewhat stronger (H_2SO_4 and $NaOH$ in strengths of $n/200$ to $n/1000$), at least so far as those experiments are concerned which could approximately be compared with mine, has apparently obtained similar results, although he interprets them differently.

Table V gives a summary of some 33 indices obtained by Oker-Blom under the influence of H_2SO_4 and 36 indices obtained under the influence of $NaOH$, which were the result of experiments carried out much on the same lines, as my own and therefore capable of comparison. The other experiments of Oker-Blom were carried out under methods so

different from my own as to preclude them from all comparison, *e.g.* the use of H_2SO_4 and NaOH in strengths of $n/40$ and $n/20$; the neutralization of the acid or alkali used for digestion before the addition of the leucocytes: digestion of leucocytes or both leucocytes and cocci with acid and alkali.

TABLE V. (*From Oker-Blom.*)

Digestion with			Average phagocytic index	
H_2SO_4	$n/200$...	186	} 185
H_2SO_4	$n/400$...	185	
H_2SO_4	$n/1000$...	186	
Physiological saline		...	100	
NaOH	$n/1000$...	114	} 112
NaOH	$n/400$...	115	
NaOH	$n/200$...	107	

There was one difference, and that in counting, between Oker-Blom's methods and my own. Oker-Blom counted what he called the "verankerte" cocci, which, as he explained in a private communication, were all cocci found in contact with the leucocytes, *i.e.* not only the ingested ones, but also those which were merely attached to the periphery of the leucocytes. Such "attached" cocci were not frequent in my slides, and to me it seems a matter of considerable difficulty to decide which cocci are attached and which are merely in juxtaposition to the leucocytes. It is very interesting that with two such dissimilar methods of counting, results comparable to the degree that they are should have been obtained.

Although Oker-Blom decides that the indices obtained under the influence of NaOH may be looked upon as showing an accelerating effect of the alkali on phagocytosis, my experience would lead me to believe that the average alkali count as given in Table V still lay within the zone of experimental error, and that the inference to be drawn was, that while acid had the effect of markedly increasing phagocytosis, alkali on the other hand had very little or no influence.

To account for an accelerating effect on the part of both acid and alkali Oker-Blom argued that as bacteria were notoriously negatively charged and as leucocytes judging by the acidophilic staining reaction of their protoplasm was obviously positively charged, there existed from the beginning a mutual attraction and one would only have to explain how by digestion with either acid or alkali this attraction was heightened. This he attempted to do as follows, supposing bacteria and leucocytes to be affected by the acid and alkali in three phases.

(1) On mixing bacteria and acid, bacteria enter *First Phase* and have a lessened negative charge.

(2) At end of digestion of bacteria and acid bacteria are in the *Second Phase*, there being a state of balance between the two; the bacteria have a neutral charge.

(3) On adding leucocytes suspended in physiological saline and thus lowering the acidity of the mixture, the bacteria enter the *Third Phase* and show a negative charge greater than the initial one, while the leucocytes suddenly finding themselves in an acid environment take on a stronger positive charge than they possessed before (*First Phase* for the leucocytes).

In the same way, apparently, when digesting bacteria with alkali, the bacteria end up with a positive charge and the leucocytes with a negative one. In both cases it is assumed that the mutual attraction between the bacteria and the leucocytes has been increased.

To test this theory of Oker-Blom, it was necessary to determine experimentally the charge possessed by both bacteria and leucocytes in neutral solutions and under the influence of acid and alkali. The attempt to demonstrate the convection of leucocytes in an electric field by the U-tube method had to be abandoned; the leucocytes proved too heavy, and sedimented before any cataphoresis could be observed. Subsequently, the glass slide method was employed for both bacteria and leucocytes, and proved very satisfactory. An ordinary glass slide has cemented to it at either end a broad, flat platinum electrode; a few drops of the suspension to be tested are placed between the electrodes and covered by a cover slip which rests on the two electrodes, thus forming a shallow cell some .2 to .3 mm. in depth, in which by the aid of a dark-ground illumination microscope cataphoresis may be observed. The electrodes were connected with the poles of a battery possessing a charge of about seven volts, a switch being interposed to allow of the current being quickly and conveniently closed, opened or reversed. As the formation of any gas at the electrodes would be fatal to a correct observation, it is impossible to carry out the experiments in physiological saline solution. For the suspension of bacteria, distilled water can of course be used, but for leucocytes, at any rate for longer observations, it is necessary to suspend in an isotonic solution. In these experiments 8.8 % cane sugar in distilled water was taken. Acid and alkali solutions similar in strength to those used in the phagocytosis experiments were prepared, in the one case distilled water, in the other isotonic sugar solution being the diluent.

Suspended in a neutral solution, the staphylococci showed a marked negative charge and this charge was not altered in the presence of either $n/1000$ or $n/100$ acetic acid, even after they had been in contact as long as 24 hours. Indeed in no way could the cocci be induced to take on a positive charge under the influence of the acid, unless there was present in the solution an amphoteric electrolyte, *e.g.* the haemoglobin that had laked out of red corpuscles—when, apparently adsorption of protein on the cocci took place. As the protein coat took on a positive charge in response to the acid, the whole coccus moved towards the negative pole.

Leucocytes, obtained either in the form of pus cells from urine or from the peritoneal cavity of the guinea-pig, in response to an injection of broth, or from citrated blood, and washed and suspended in isotonic sugar solution, show a decided negative charge, moving, on the current being closed, smartly towards the anode.

Leucocytes in the presence of red cells assume under the influence of even $n/1000$ acetic acid a positive charge, but this occurs for the same reason as that advanced for the giving of a positive charge to cocci—viz. that they are coated with adsorbed protein.

Michaelis and Takahashi (1910) have shown that by increasing the hydrogen-ions in a red corpuscle suspension beyond 1×10^{-5} , the red cells are laked. Acetic acid $n/1000$ represents $[H^+] = 0.13 \times 10^{-3}$. When blood corpuscles are suspended in $n/1000$ acetic acid, the red cells lose haemoglobin which, by coating the leucocytes, gives them a positive charge. How the leucocytes would behave without red cells present to yield haemoglobin does not concern one here, as red corpuscles are always present in phagocytic systems such as those under consideration. As was to be expected, both cocci and leucocytes retain their negative charge in the presence of alkali, ammonia up to $n/100$ being used. On bringing a mixture of cocci and blood corpuscles, all previously showing a negative charge by moving in an electric field towards the anode, to $n/1000$ with acetic acid, the charge of both the cocci and the blood corpuscles becomes a positive one, migration taking place towards the kathode. The addition of ammonia in the place of the acid leaves the bodies negative as they were before.

The facts gained from these observations would seem to point to the following explanation of the results obtained in the experiments on spontaneous phagocytosis.

If the fact of bacteria and leucocytes possessing an electric charge of their own is going to play a part in phagocytosis, and explain or help

to explain why it is that on the spontaneous phagocytosis of staphylococci acid exerts an accelerating influence and alkali either none at all or a retarding one, it is obviously because both cocci and leucocytes possess, in the neutral control experiments, a negative charge which is either unaltered or somewhat increased by alkali, and decreased or altered to positive by acid.

With alkali present one would thus suppose phagocytosis, *i.e.* the coming together of coccus and leucocyte, to be confronted with difficulties similar to, if not greater than, those met with in the control experiments. The coccus and the leucocyte charged with electricity of the same sign, to the same or perhaps a higher degree than in a neutral solution, will tend to keep apart to the same or even greater extent, *i.e.* phagocytosis will be the same as in the control experiments or will be retarded.

With acid present, unless the electric charge of the cocci and the leucocytes is carried as far on the positive side as it is on the negative side in a neutral solution, the forces tending to keep the two apart must be less, *i.e.* phagocytosis will be accelerated.

Before applying this hypothesis to the phagocytic experiments recorded earlier in this paper, it is necessary to examine the leucocytes and cocci in the actual phagocytic system, *i.e.* using the same thick emulsions as are used in spontaneous phagocytosis. It is not to be expected that such marked changes of charge will be observable, seeing that the absorption of acid and alkali by the large number of corpuscles and cocci present, will have lowered the hydrogen-ion concentration of the mixture very considerably.

The usual thick staphylococcus emulsion and washed corpuscles and acid and alkali solutions were then taken, the only difference being that in every case isotonic sugar solution was used instead of physiological saline in order to avoid disturbance in the electric cell. Equal volumes of staphylococcal emulsion and acetic acid $n/50$ and $n/300$, and ammonia $n/50$ and $n/300$, and isotonic sugar solution (this in place of the usual physiological saline control) were digested for half an hour at room temperature, then third and equal volumes of corpuscles added and the whole incubated in the shaker at 37° C. for half an hour. At the end of this time the various mixtures were examined for cataphoresis in the electric cell, and as they were much too thick for direct examination, a preliminary centrifuging to sediment the corpuscles and most of the cocci was necessary.

Taking the supernatant and a few of the corpuscles it was possible to observe the charge possessed by the leucocytes and by the cocci.

In all cases however, whether phagocytosis had taken place under the influence of acid or alkali or neither, the cocci and corpuscles moved towards the positive pole, nor did the rate at which they moved appear to differ to any extent. There was therefore no proof here that the electric condition of the cocci and leucocytes had been greatly altered by the addition of acid or alkali to the system. The hydrogen-ion content probably differs so little in these final mixtures that a difference in charge cannot be demonstrated by cataphoresis in the electric cell. Yet from this one may not conclude that no alteration at all in the amount of negative charge possessed by leucocytes and cocci has taken place. Any change in charge brought about by either acid or alkali would necessarily be a very much smaller one and so very likely to escape detection in the rather rough estimation possible in the electric cell. What the charge of a particle may be is easily decided, but whether it is a little more or a little less negatively charged is a difficult question to decide.

To determine exactly what the hydrogen-ion concentration of these phagocytic systems is, thick emulsions of blood corpuscles and cocci were prepared and acid, alkali or saline added as was done in the experiments detailed earlier in the paper. In these mixtures estimations were carried out by the gas-chain method and it was apparent to what a very considerable extent both acid and alkali had been absorbed. In every case the mixture was on the alkaline side of the neutral point. The addition of acid, even in the strength of $n/50$, did not suffice to give an acid reaction. With a lowering of the amount of alkali added or an increase in the amount of acid a rise in the hydrogen-ion concentration took place but the range of alteration was very small, the hydrogen-ion concentration being in the phagocytic system to which $n/50$ ammonia had been added $10^{-7.72}$ normal and in that to which $n/50$ acetic acid had been added $10^{-7.21}$ normal.

In thick emulsions of corpuscles and cocci as used in spontaneous phagocytosis there is always a certain amount of protein present in solution and, judging by those cataphoresis experiments where the hydrogen-ion content was greater, it is to be concluded that the negative charge of the cocci and leucocytes has sunk with the rise in the hydrogen-ion concentration of the mixture, although not to a sufficient extent to be noticeable in a cataphoresis experiment, and indeed the small extent to which the hydrogen-ions are increased or decreased by the addition of acid or alkali, would not lead one to expect a greatly altered charge.

It is to be supposed that in a phagocytic system alterations of charge differing in degree, though similar in character to those demonstrable when the proportion of acid to cocci and leucocytes in the emulsion is greater, have occurred, and that the lowering of the negative charge of cocci and leucocytes by acid may help to explain the acceleration of phagocytosis observed to have been caused by acetic acid, while the addition of alkali to a phagocytic system may retard phagocytosis by increasing the negative charge of the cocci and leucocytes.

CONCLUSIONS.

(1) Spontaneous phagocytosis appears, like normal phagocytosis occurring in the presence of serum, to be a process of an adsorptive nature, but owing to the wide range of experimental error this is not capable of exact proof as is the case with phagocytosis in the presence of serum.

(2) The spontaneous phagocytosis of staphylococci taking place on the addition of acid (acetic acid $n/50$ to $n/330$) undergoes marked acceleration, the average count for all experiments thus carried out being about 80 % higher than when no acid was present.

(3) Alkali (ammonia $n/50$ to $n/330$) affects spontaneous phagocytosis in weaker solutions scarcely at all; in stronger dilutions ($n/50$) it retards the ingestion of the cocci, the experiments performed giving an average count about 25 % lower when the stronger alkali was used than when alkali was absent.

(4) In neutral and alkaline suspensions both cocci and leucocytes possess a negative electric charge.

(5) In acid suspensions with haemoglobin or other amphoteric electrolyte present in solution cocci and leucocytes take on a positive charge, this being apparently due not to a direct effect of hydrogen-ions on the cocci and leucocytes, but to their action on the coat of protein these bodies had obtained by adsorption of the haemoglobin in solution.

(6) In a phagocytic system a certain amount of protein is free in solution, and it is suggested that, on the addition of acid, the cocci and leucocytes, owing to their possessing adsorbed protein coats, lose a certain amount of their negative charge, while on the addition of alkali the charge is somewhat raised, the extent to which this occurs however being small, as the addition of even $n/50$ acetic acid or ammonia to a phagocytic system results in only a small alteration in the $[H^+]$ concentration (the major portion of the acid and alkali being absorbed by

the protein of the corpuscles and cocci). This alteration of charge would explain why acid, by causing a decrease in the forces tending to keep the cocci and leucocytes apart, raises the phagocytic index, while alkali, by bringing about an increase in these forces, lowers the phagocytic index.

(7) Experiments carried out with the actual emulsions of cocci and leucocytes similar to those used in spontaneous phagocytosis have failed to show a decrease of negative charge in the cocci and leucocytes on addition of acetic acid up to $n/50$ or an increase under the influence of ammonia. It is possible that the alterations in charge in these cases are too small to be observable in a cataphoresis experiment and yet sufficiently large to influence phagocytosis.

I wish to express my indebtedness to Dr Ledingham and Dr Harriette Chick for much valuable assistance given in the course of this research.

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ON THE NATURE OF BACTERIAL LAG.

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(With 5 Charts.)

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Introduction.

THE rate of bacterial growth was first measured with any degree of accuracy in the case of the *Vibrio cholerae asiaticae* growing in broth, by Buchner, Longard and Riedlin (1887). They gave a generation time in the case of this organism of 19 to 40 minutes. These times were calculated from the numbers of those inoculated and of those found to be present in cultures after two to five hours' growth.

The large differences obtained in different experiments were explained by variations of the strain in artificial culture. Max Müller (1895) drew attention to the fact that the wide variations in the figures of Buchner, Longard and Riedlin were due, not as these authors believed to variations of the strain, but to the fact that the periods of observations varied in length; he pointed out that those cultures which gave long generation times had been allowed to grow only for short periods, while those which gave short generation times had been allowed to grow for longer periods. Further, this author, by a series of counts, was able to demonstrate experimentally the existence of lag.

By bacterial lag, we understand, the interval between the inoculation of a bacterial culture and the time of commencement of its *maximum* rate of growth. This has also been referred to as latency, restraint of growth, and various other terms.

The measurement of lag.

(1) The lag may be expressed in terms of the period (hours) during which submaximal growth continues.

(2) Myer Coplans (1909) has expressed it as restraint of growth in terms of minimum generation times. If, for example, x hours were required after inoculation before a culture showed its minimum generation time, and if it then had a generation time of y hours, and the number of generations that actually arose during x was z then $\frac{x - zy}{y}$ = the measure of restraint of growth in terms of minimum generation times.

(3) An index of lag is readily obtained by comparing the average generation time during the first hours of growth with the average generation time of a succeeding period or several such periods.

All these methods have difficulties. The error of measurement of numbers of bacteria present is not inconsiderable, and this circumstance makes the precise definition of the limits of lag difficult.

Where attempts are made to estimate generation time on an increase of bacterial population amounting to 0.3 of a generation the results are very unreliable. On the other hand, where two generations or more have developed during the period under consideration, results of great precision can be obtained. The investigation of lag involves a large number of experiments of a comparative character. To diminish the number of variables it is advisable to use the same sample of peptone

in preparing the peptone-water medium. The temperature must be carefully noted several times during the continuance of each experiment. The organism of course is a factor that we cannot yet keep constant, for which reason it is desirable when endeavouring to ascertain the influence of any one factor on lag that all the comparative experiments be done simultaneously.

Lag is a subject of considerable interest. It occurs in many biological reactions, for example, haemolysis, bacteriolysis and many others, and it seems not impossible that light thrown on any one of these may illumine the rest. Further, the incubation period of infectious disease may partly depend for its existence on bacterial lag.

Satisfactory quantitative work on this subject is small in amount. I intend therefore to submit records of experiments showing in a quantitative manner the influence of various factors on lag.

Technique.

The culture medium used was always 1.0 % peptone (Witte) + 0.5 % salt. It was sterilized by autoclaving, and its reaction was faintly alkaline to neutral litmus paper. *B. coli* was the organism employed. It was subcultured every day from peptone water to peptone water of the composition indicated and used generally as a 17 to 20 hours' culture for the inoculation of the peptone water of the actual experiment. The actual experiments were always carried out at 37° C. unless otherwise stated. The parent cultures were likewise always grown at 37° C. The standard drop of the experiments was 0.02 c.c.

The agar plating method was used to ascertain the numbers of bacteria in the growing cultures. A small amount of agar was first poured into each plate. When this had set, the quantity of bacterial emulsion was added to the plate and then a whole tube of agar added and mixed with the emulsion. After about five minutes, a further small quantity of agar was added, sufficient to cover the second layer. This method greatly facilitated the counting process as all colonies were discrete, and no spreading occurred. All dilutions of parent cultures and subcultures were made in normal saline solution.

In the following experiments generation time signifies the average generation time during the interval dealt with. If, during the interval, the generation time has been varying, this method of expression is not entirely satisfactory.

In such cases generation time at particular moments is the only absolutely satisfactory expression. The former method has however

been largely and legitimately used in recorded work. Comparatively few of the recorded experiments on this subject enable one to obtain the generation time, with any accuracy, at particular moments during periods of varying rate of growth, and this precision is not usually necessary. (See Ledingham and Penfold, *This Journal*, p. 242.)

Factors influencing lag.

Size of inoculum. Size of inoculation was stated by Rahn (1906) to have an influence on lag. He stated that the greater the inoculum the shorter the lag. Since this appears to have an important bearing on the nature of lag, I examined his evidence carefully. Rahn's figures however will not bear careful scrutiny. In Table I I reproduce his numbers, his experimental data and generation times. It will be noticed that in columns III, IV and V the initial count per c.c. increases from III to V while the volumes are constant. His calculated generation times, given below, suggest that the maximum lag is obtained in III but this is found to depend on an arithmetical error since, on recalculation, tube III in reality shows its minimum generation time in the same time interval as tube IV. I have introduced certain corrections in arithmetic into his table, these corrections being underlined. In the case of tube V its lag is shorter than III and IV, but it never attains

TABLE I. [*Tabelle III (Page 422).*]

Bacillus fluorescens in Traubenzuckerpeptonlösung. Bakterienanzahl pro c.cm.

	I	II	III	IV	V
Flüssigkeitsmenge	1000 c.cm.	10 c.cm.	100 c.cm.	100 c.cm.	100 c.cm.
Am Anfang	30,000	30,000	3,000	30,000	3,000,000
Nach 6 Stunden	50,500	—	[200]	—	5,280,000
„ 12 „	290,800	7,610,000	[200]	330,000	20,000,000
„ 24 „	6,430,000	82,000,000	130,000	33,000,000	59,000,000
„ 36 „	10,000,000	180,000,000	13,000,000	70,000,000	82,000,000
„ 53 „	8,000,000	75,000,000	66,000,000	110,000,000	66,000,000

Generationsdauer.

	I	II	III	IV	V
von 0-6 Stunden	479 mins.	90 mins.	[∞] mins.	210 mins.	462 mins.
„ 6-12 „	1616 „ <u>142</u>		[∞] „ }		187 „
„ 12-24 „	61 „ <u>161</u>	210 „	132 „ <u>77</u>	108 „	461 „
„ 24-36 „	1130 „	634 „	108 „	664 „	1629 „
„ 36-53 „	∞	∞	435 „	1564 „	∞

The underlined numbers are corrections of arithmetic which I have introduced.

very rapid growth and its short lag is probably really due to the fact that the factors inhibiting growth came into operation before the lag had ceased. The series therefore on which Rahn relied will not bear examination.

It is extremely interesting to note in the same table that I, II and IV form a comparable series to which Rahn did not draw attention. They all have the same initial number per c.c. but, owing to the large difference in volume, the size of inoculum is enormously different. The time figures given after again revising the arithmetic, show that the smallest inoculum has the shortest lag, an opposite conclusion to that drawn by Rahn. A further serious drawback to Rahn's work is the infrequency of observations. It is quite obvious that to establish the minimum generation time of a culture satisfactorily, two, or better still, three, consecutive periods with approximately the same generation time, within the limits of experimental error, are required, or the several tubes of the same strain growing in the same medium should show the same generation time proving that they have attained comparable rates of growth. Rahn's work on the point does not satisfy either of these conditions. The minimum generation times which he found in the five tubes of the same culture medium inoculated with the same organism at the same time, varied widely, as a reference to Table I will immediately show.

The position required further experimental work. For this purpose I tested the effect of size of inoculum on the lag shown by *B. coli* Escherich when grown on peptone water. Table II gives one complete experiment. The experiment was conducted in duplicate and the number per drop determined on the average of the two plates. The sizes of inoculum were approximately as 1 : 10, 25 : 100. The tubes were warmed to 37° C. before inoculation. The volumes were constant and all the tubes were put in and out of the incubator together, they are strictly comparable. Chart 1 shows the logarithms of the various numbers plotted against time and it is seen that the curves are very similar in each case.

In Table III are shown the average generation times for the first two hours as against those for the third hour. It will be observed that during the third hour the generation times in the case of all the tubes are constant within the error of experiment, while in the case of the first two hours, the generation time diminishes slightly as we pass from the smaller to the larger seedings.

In Table IV are given the average generation times of the first two hours averaged from two experiments each conducted in duplicate,

TABLE II. EXPERIMENT A. *The effect of Size of Inoculum on Lag.*

A = 9 c.c. peptone water inoculated with 6 drops of $\frac{1}{100}$ dilution of a 20 hours' culture of <i>B. coli</i> on peptone water. <div> <div>All peptone water warmed to 37° C. before inoculation.</div> <div>Temperature of incubator during this experiment = 36.2° C.</div> </div>									
Tubes	0	45 mins.	60 mins.	80 mins.	100 mins.	120 mins.	150 mins.	180 mins.	Generation times, min.
A ₁	2 drops neat = 426	2 drops neat = 597	1 drop neat = 323	1 drop neat = 472	5 drops of $\frac{1}{10}$ dil. = 342	5 drops of $\frac{1}{20}$ dil. = 324	4 drops of $\frac{1}{40}$ dil. = 268	4 drops of $\frac{1}{80}$ dil. = 390	0 to 120 45
A ₂	2 " = 444	2 " = 550	1 " = 367	1 " = 468	5 drops ditto = 376	5 drops ditto = 357	4 drops ditto = 239	4 drops ditto = 371	120 to 180 24 150 to 180 19
	Average per neat drop = 217.5 Log = 2.33746	Average per neat drop = 287 Log = 2.45788	Average per neat drop = 345 Log = 2.53782	Average per neat drop = 470 Log = 2.67210	Average per neat drop = 718 Log = 2.85612	Average per neat drop = 1362 Log = 3.13418	Average per neat drop = 2535 Log = 3.40398	Average per neat drop = 7610 Log = 3.88138	
B ₁	6 drops neat = 355	6 drops neat = 454	6 drops neat = 671	4 drops neat = 562	3 drops neat = 554	1 drop neat = 345	5 drops of $\frac{1}{10}$ dil. = 402	5 drops of $\frac{1}{20}$ dil. = 315	0 to 120 47
B ₂	6 " = 358	6 " = 507	6 " = 595	4 " = 562	3 " = 583	1 " = 363	5 drops ditto = 387	5 drops ditto = 335	120 to 180 24
	Average per neat drop = 59.4 Log = 1.77379	Average per neat drop = 80 Log = 1.90309	Average per neat drop = 105.5 Log = 2.02325	Average per neat drop = 140.5 Log = 2.14768	Average per neat drop = 189.5 Log = 2.27761	Average per neat drop = 354 Log = 2.54900	Average per neat drop = 789 Log = 2.89708	Average per neat drop = 1950 Log = 3.29003	
C ₁	20 drops neat = 379	20 drops neat = 609	20 drops neat = 699	15 drops neat = 685	10 drops neat = 722	6 drops neat = 658	15 drops of $\frac{1}{10}$ dil. = 339	5 drops of $\frac{1}{20}$ dil. = 305	0 to 120 48
C ₂	20 " = 431	20 " = 593	20 " = 708	15 " = 787	10 " = 755	6 " = 715	15 drops ditto = 381	5 drops ditto = 316	120 to 180 24-6
	Average per neat drop = 20.2 Log = 1.30535	Average per neat drop = 30 Log = 1.47712	Average per neat drop = 35.2 Log = 1.54654	Average per neat drop = 49.1 Log = 1.69108	Average per neat drop = 73.8 Log = 1.86806	Average per neat drop = 114.4 Log = 2.05843	Average per neat drop = 240 Log = 2.38021	Average per neat drop = 621 Log = 2.79309	
D ₁	$1\frac{1}{2}$ c.c. = 176	$1\frac{1}{2}$ c.c. = 254	$1\frac{1}{2}$ c.c. = 327	1 c.c. = 283	40 drops neat = 269	25 drops neat = 258	9 drops neat = 238	3 drops neat = 202	0 to 120 56
D ₂	$1\frac{1}{2}$ c.c. = 189	$1\frac{1}{2}$ c.c. = 267	$1\frac{1}{2}$ c.c. = 353	1 c.c. = 278	40 " = 302	25 " = 273	9 " = 213	3 " = 201	120 to 180 22
	Average per neat drop = 2.4 Log = 0.38021	Average per neat drop = 3.5 Log = 0.54407	Average per neat drop = 4.5 Log = 0.65321	Average per neat drop = 5.6 Log = 0.74819	Average per neat drop = 7.1 Log = 0.85126	Average per neat drop = 10.6 Log = 1.02531	Average per neat drop = 25 Log = 1.39794	Average per neat drop = 67 Log = 1.82607	

from which it will be seen the larger inoculum definitely tends to grow a little better during the initial period.

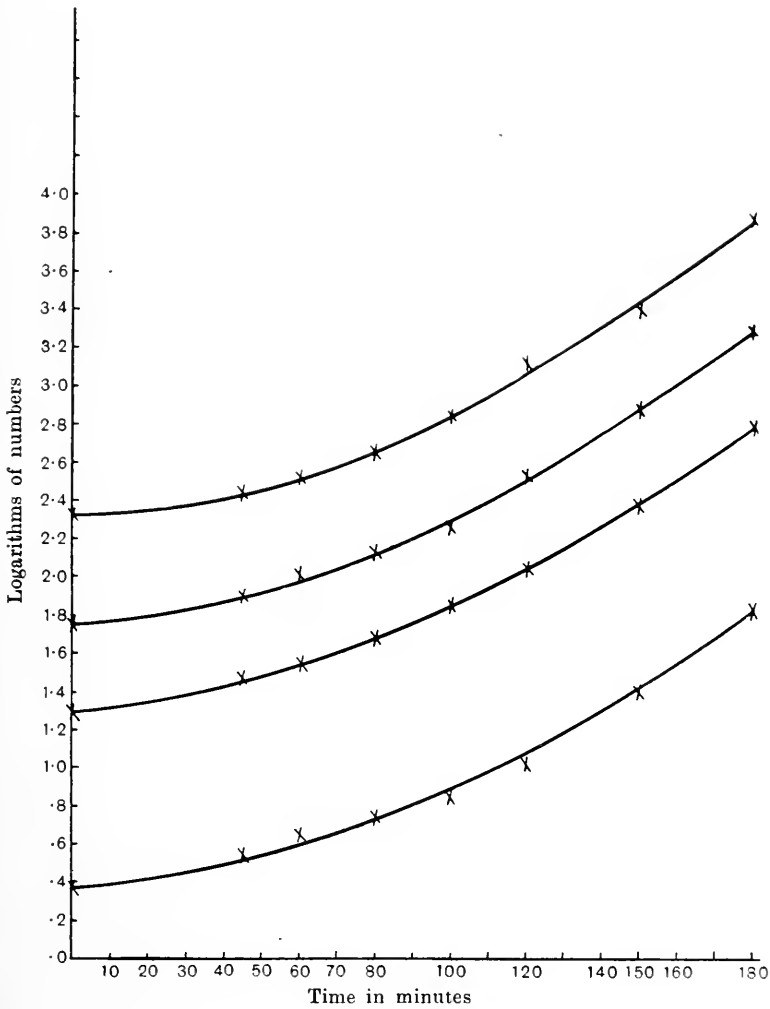


Chart 1. The effect of size of inoculation on lag experiments.

TABLE III.

Dilution of parent culture—			$\frac{1}{10000}$	$\frac{1}{1000}$	$\frac{1}{400}$	$\frac{1}{100}$
No. of bacteria per c.c. inoculated			(120)	(1010)	(2970)	(10875)
First 2 hours	56	48	47	45 mins.
2nd to 3rd hour	22	24.6	24	24

TABLE IV. *The generation times of cultures with different inoculums, from two experiments, for the first two hours of growth, with their averages.*

Dilution of parent culture—	$\frac{1}{100}$	$\frac{1}{400}$	$\frac{1}{1000}$	$\frac{1}{10000}$
Experiment B ...	44	48	52	91
.. A ...	45	47	48	56
Average of A and B	44.5	47.5	50	73.5

The details of Experiment B, which was carried out on exactly similar lines to Experiment A, are shown in Table V.

The points in these two experiments were determined on large counts so that the figures might be used for the mathematical treatment of this early portion of the growth curve. (See Ledingham and Penfold, *This Journal*, p. 242.)

Employment of larger seedings.

An endeavour was made to elucidate the effect of size of inoculum on lag, in the case of much bigger seedings. The initial population in the case of the largest reached nearly 200,000 per c.c.

One such experiment is recorded in Table VI. This, taken in conjunction with the protocols of the preceding experiments on the same subject, shows that as the inoculum is increased, the diminution of the lag becomes less and ultimately practically disappears. The generation times of the A and B experiments of Table IV for the preliminary two hours of cultures from dilution $\frac{1}{100}$ are practically identical with that obtained in the case of the corresponding culture from $\frac{1}{1000}$ dilution in Experiment C, Table VI, so that these experiments may be reasonably looked upon as one. The experiments were not carried further than a three hours' observation period because, as is seen in Table II, the minimum generation time was attained during the last half hour in each case within the experimental error, viz. 19 to 23 minutes.

Effect on lag of sudden chilling of a culture growing at maximum pace.

The object of these experiments was to see if stoppage by cold, of growth at its maximum, was followed by a lag, when the temperature of the culture was raised again suddenly to its original height. *B. coli* was the organism used and peptone water the medium. Three cultures

TABLE V. EXPERIMENT B. *The effect of Size of Inoculum on Log.*

A = 9 c.c. of peptone water inoculated with 6 drops of $\frac{1}{100}$ dilution of 20 hours' culture of <i>B. coli</i> on peptone water.									
<div> <div>Incubated at 37° C. All peptone water warmed to 37° C. before inoculation.</div> <div></div> </div>									
Tubes	0	40 mins.	60 mins.	80 mins.	100 mins.	120 mins.	150 mins.	180 mins.	Generation times, mins.
A ₁	2 drops neat = 262	2 drops neat = 353	1 drop neat = 214	1 drop neat = 356	5 drops of $\frac{1}{100}$ dil. = 272	5 drops of $\frac{1}{200}$ dil. = 229	4 drops of $\frac{1}{100}$ dil. = 166	4 drops of $\frac{1}{200}$ dil. = 251	0 to 120
A ₂	2 " = 314	2 " = 327	1 " = 231	1 " = 314	5 drops ditto = 261	5 drops ditto = 254	4 drops ditto = 211	4 drops ditto = 333	0 to 120
	Average per drop = 144	Average per drop neat = 170	Average per drop neat = 222	Average per drop neat = 335	Average per drop neat = 533	Average per drop neat = 966	Average per drop neat = 1885	Average per drop neat = 5840	120 to 180
	Log = 2.15336	Log = 2.23045	Log = 2.34635	Log = 2.52504	Log = 2.72673	Log = 2.98498	Log = 3.27531	Log = 3.76641	150 to 180
B ₁	6 drops neat = 227	6 drops neat = 286	6 drops neat = 351	4 drops neat = 341	3 drops neat = 404	1 drop neat = 161	5 drops of $\frac{1}{100}$ dil. = 231	5 drops of $\frac{1}{200}$ dil. = 191	0 to 120
B ₂	6 " = 201	6 " = 343	6 " = 351	4 " = 354	3 " = 375	1 " = 237	5 drops ditto = 241	5 drops ditto = 267	120 to 180
	Average per drop neat = 35.7	Average per drop neat = 53.4	Average per drop neat = 58.5	Average per drop neat = 87	Average per drop neat = 130	Average per drop neat = 199	Average per drop neat = 472	Average per drop neat = 1194	150 to 180
	Log = 1.55267	Log = 1.71933	Log = 1.76716	Log = 1.93952	Log = 2.11394	Log = 2.29885	Log = 2.67394	Log = 3.07700	150 to 180
C ₁	20 drops neat = 287	20 drops neat = 263	20 drops neat = 390	15 drops neat = 382	10 drops neat = 373	6 drops neat = 375	15 drops of $\frac{1}{100}$ dil. = 191	5 drops of $\frac{1}{100}$ dil. = 167	0 to 120
C ₂	20 " = 276	20 " = 315	20 " = 423	15 " = 421	10 " = 447	6 " = 438	15 drops ditto = 263	5 drops ditto = 204	0 to 120
	Average per drop neat = 14	Average per drop neat = 15.2	Average per drop neat = 20.3	Average per drop neat = 26.8	Average per drop neat = 41	Average per drop neat = 67.8	Average per drop neat = 151.3	Average per drop neat = 371	120 to 180
	Log = 1.14613	Log = 1.18181	Log = 1.30750	Log = 1.42813	Log = 1.61278	Log = 1.83123	Log = 2.17984	Log = 2.56337	150 to 180
D ₁	$1\frac{1}{2}$ c.c. = 126	$1\frac{1}{2}$ c.c. = 131	$1\frac{1}{2}$ c.c. = 113	1 c.c. = 149	1 c.c. = 172	25 drops neat = 103	9 drops neat = 80	3 drops neat = 69	0 to 120
D ₂	$1\frac{1}{2}$ c.c. = 134	$1\frac{1}{2}$ c.c. = 132	$1\frac{1}{2}$ c.c. = 139	1 c.c. = 106	1 c.c. = 137	25 " = 108	9 " = 76	3 " = 82	120 to 180
	Average per drop neat = 1.7	Average per drop neat = 1.75	Average per drop neat = 1.88	Average per drop neat = 2.25	Average per drop neat = 3.09	Average per drop neat = 4.22	Average per drop neat = 8.66	Average per drop neat = 25.16	150 to 180
	Log = 0.23045	Log = 0.24304	Log = 0.27416	Log = 0.35218	Log = 0.48996	Log = 0.62531	Log = 0.93752	Log = 1.40071	150 to 180

were inoculated from a dilution in saline of a 17 hours' peptone water culture of *B. coli* and all were grown at 37° C.

(1) The first was kept at 37° C. throughout as control.

(2) The second culture, after it had grown two hours, was chilled for twelve minutes at 2° C.; it was then heated suddenly during two minutes in a water bath up to 37° C. and plated, after which the containing tube was quickly dried and placed in the incubator at 37° C. Plating of samples followed from time to time. The sudden cooling stopped growth but on being heated again to 37° C. maximum growth was resumed without lag.

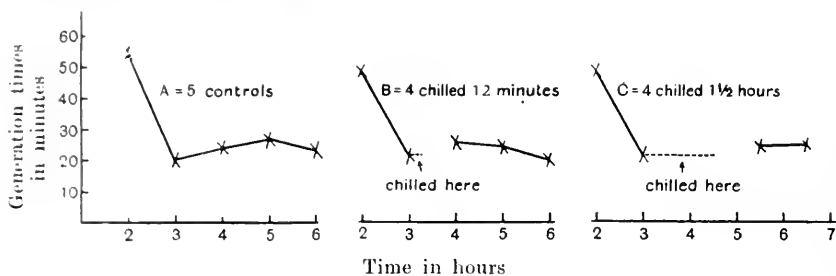


Chart 2a. Chilling during maximum growth and lag.

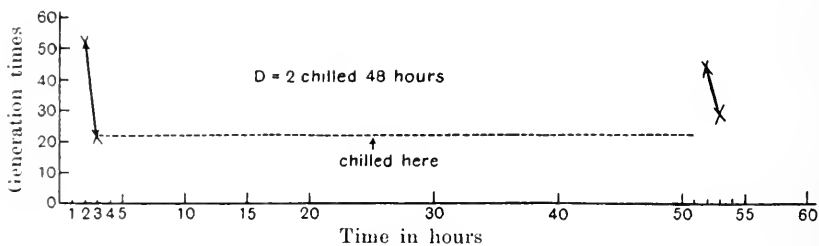


Chart 2b.

(3) The third culture was cooled for a longer period, viz. one hour and a half. On being heated again to 37° C., it grew entirely without lag during the first hour. Five such experiments were done. One is recorded in full detail in Table VII. A résumé of four is given in brief in Table VIII and from these figures Chart 2 has been constructed.

The fifth chart was not considered in making the averages as it was an early experiment and did not show all the necessary data.

In two of the experiments Culture III, during the process of chilling, was halved and one half placed in the refrigerator till the 2nd following day. It was then again suddenly heated to 37° C. and counted by

TABLE VI. *The effect of Size of Inoculum on Lag. (Larger Seedings.)*

		Generation times, mins.					
		0	60 mins.	90 mins.	120 mins.	150 mins.	180 mins.
A. $\frac{1}{2}$							
A = 3 drops neat of a 20 hours' peptone water culture of <i>B. coli</i> inoculated into 9 c.c. of peptone water.							
B=6	" of $\frac{1}{2}$ dil. of "			"	"	"	Incubated at 37° C.
C=6	" of $\frac{1}{10}$ dil. of "		"	"	"	"	
All peptone water warmed to 37° C. before inoculation. N.B. instead of having A=6 drops of $\frac{1}{2}$ dilution 3 drops neat were used.			"	"	"	"	
A ₁ 2 drops of $\frac{1}{3}$ dil. = 141		1 drop of $\frac{1}{3}$ dil. = 95	1 drop of $\frac{1}{10}$ dil. = 104	1 drop of $\frac{1}{30}$ dil. = 124	1 drop of $\frac{1}{90}$ dil. = 99	1 drop of $\frac{1}{270}$ dil. = 73	
A ₂ 2 drops ditto = 166		1 drop ditto = 111	1 drop ditto = 111	1 drop ditto = 88	1 drop ditto = 88	1 drop ditto = 84	
Average per neat drop = 3837.5		Average per neat drop = 5150	Average per neat drop = 9600	Average per neat drop = 31800	Average per neat drop = 82350	Average per neat drop = 240000	0 to 120 = 39.3
Log = 3.58405		Log = 3.71181	Log = 3.98227	Log = 4.50243	Log = 4.91566	Log = 5.38021	120 to 180 = 20
B. $\frac{1}{10}$ th							
B ₁ 2 drops of $\frac{1}{10}$ dil. = 158		1 drop of $\frac{1}{10}$ dil. = 96	1 drop of $\frac{1}{10}$ dil. = 98	1 drop of $\frac{1}{30}$ dil. = 101	1 drop of $\frac{1}{90}$ dil. = 55	1 drop of $\frac{1}{270}$ dil. = 64	
B ₂ 2 drops ditto = 124		1 drop ditto = 109	1 drop ditto = 109	1 drop ditto = 90	1 drop ditto = 91	1 drop ditto = 76	0 to 120 = 39.6
Average per neat drop = 352.5		Average per neat drop = 512.5	Average per neat drop = 940	Average per neat drop = 2880	Average per neat drop = 5400	Average per neat drop = 17500	120 to 180 = 23
Log = 2.54716		Log = 2.70969	Log = 2.97313	Log = 3.45939	Log = 3.73239	Log = 4.24304	
C. $\frac{1}{10}$ th							
C ₁ 2 drops neat = 195		1 drop neat = 128	5 drops of $\frac{1}{10}$ dil. = 128	5 drops of $\frac{1}{30}$ dil. = 101	5 drops of $\frac{1}{90}$ dil. = 84	5 drops of $\frac{1}{270}$ dil. = 82	0 to 120 = 45
C ₂ 2 " = 173		1 " = 130	5 drops ditto = 113	5 drops ditto = 92	5 drops ditto = 72	5 drops ditto = 63	120 to 180 = 23
Average per neat drop = 92		Average per neat drop = 129	Average per neat drop = 241	Average per neat drop = 579	Average per neat drop = 1404	Average per neat drop = 3625	
Log = 1.96379		Log = 2.11059	Log = 2.38202	Log = 2.76268	Log = 3.14737	Log = 3.55931	

plating. Immediately thereafter it was placed in the incubator, and counted again after one hour and two hours' growth respectively. In this case the numbers are small, especially of the last count, but in the case of the first and second counts, see Table IX and Chart 2 b, they are sufficient to show that the lag has reappeared, the generation times during the first hour after chilling being 44 minutes while in the case of the evanescent chilling only 21 and 24 minutes were required for one generation during the same interval. *We may therefore state that if maximum growth be inhibited by a short application of cold it will recommence immediately without lag on the cold being removed. If, on the other hand, the cold be long continued the lag tends to reappear.*

It appeared possible that the mere stoppage of growth in parent cultures might be of itself sufficient to introduce lag, but that possibility is negatived by these experiments. The averages of the experiments on this subject are given in Table VIII.

TABLE VIII. *Experiment to show the average generation time during different intervals of peptone water cultures of B. coli Esch., 5 allowed to grow freely at 37° C. as controls and 10 subjected to chilling.*

	Average generation time in mins.				
	During first two hours of growth	During third hour of growth	4th hour	5th hour	6th hour
5 controls	55.4	20.6	24.6	27	23.5
4 cultures cooled 12 mins. each	48.5	21.75	25.7	24	20
4 cooled 1½ hours	48.5	21.25	24.5	25	
2 cooled 2 days	52	22	44	29	

The double line indicates the time of application of cold.

Subculture when growth is maximum.

The effect of subculture when growth is occurring at maximum pace has been investigated by several writers.

Rahn (1906) states that lag occurs under these conditions but adduces no satisfactory evidence in support of the assertion.

Myer Coplans (1909) agrees with Rahn's view that lag follows subculture during maximum growth. He bases his opinion on the fact that when he subcultured a parent culture of *B. coli* of 14½ hours' growth (see Chart P in the author's paper) a lag occurred. He further states that a peptone water culture of *B. coli* of 12 hours' age whether

grown at 20° or 37° C. is still in maximum development. Such a generalization is not permissible since the size of inoculation and age of parent culture and other factors must be considered in each special case.

It will also be seen from consulting Table p.p. 1 of his paper, page 4, that that assertion did not hold in this case for the average generation time between 11½ and 14½ hours is nearly twice as long as that between 8 hours and 11½ while the generation time at the end of this interval would be longer still. Without therefore direct evidence of the rate at which the parent culture grew after removal of the sample this question cannot be satisfactorily decided.

I have made six such experiments, the details of two of which are given on the annexed Table X. *B. coli* and peptone water were again used. The total volumes of the cultures were 6 c.c. The temperature of the experiments was 37° C.

The following points emerge from the consideration of Table X. The parent culture had an average generation time during the first two hours of 47 minutes, from then onwards it had 17, 23 and 21 minutes during successive intervals of approximately one hour, an average of 20.3 minutes.

The first subculture from it showed in its first three hourly intervals 20, 22 and 19 minutes respectively, that is, an average generation time of 20.3 minutes, and it is to be observed in this case that no diminution of the generation time occurred during the second hour of the development of this subculture, but its rate was identical with the first hour of its growth within the error of the experiment.

From the parent culture a second subculture was made three hours five minutes after its inoculation when it was found that the parent and the subculture grew at similar rates for both the first and second following hours.

Since all the six experiments behaved in this way I suggest that in the case of both Rahn's and Coplans' work the restraint found was due to the fact that the parent culture had really passed its period of maximum growth.

Chart 3 shows, in the case of the experiments detailed, the logarithms of the parent culture and its two subcultures plotted against time. In some of these experiments it was found that the generation time of the subculture during the first quarter of an hour appeared slightly longer than the average minimum, but it was also found to be occasionally shorter, so that it seems inadvisable to lay too great stress on rates of growth calculated from these relatively short periods. If Coplans'

TABLE IX. *An experiment to ascertain whether prolonged chilling induces lag in cultures of B. coli, chilled during maximum growth.*

The experiment was conducted in duplicate.

The original volume of the cultures was $1\frac{1}{2}$ c.c. After free growth for three hours, each was suddenly cooled in ice water, then placed in refrigerator for 48 hours and then suddenly warmed to 37° C. in a water bath.

D. The cultures in question were inoculated at the commencement of the experiment with 1 drop of $\frac{1}{400}$ dilution of an overnight culture of *B. coli* in peptone water. 1 drop = 0.02 c.c.

0 hrs.	2 hrs.	3 hrs.	51 hrs.	52 hrs. 3 mins.	53 hrs. 5 mins.	Generation times
D. 8 drops neat = 315	10 drops of $\frac{1}{4}$ dil. = 478	8 drops of $\frac{1}{40}$ dil. = 253	76 drops of $\frac{1}{40}$ dil. = 266	75 drops of $\frac{1}{200}$ dil. = 142	56 drops of $\frac{1}{400}$ dil. = 67	0 to 2 hours = 52 mins. 2 to 3 " = 22 " " " " " " " " " " " "
1 drop neat = 39.4	1 drop neat = 191.2	1 drop neat = 1.265	1 drop neat = 140	1 drop neat = 379	1 drop neat = 1675	51 to 52.3' = 44 " " " " " " " " " " " "
Log = 1.59550	Log = 2.28149	Log = 3.10209	Log = 2.14613	Log = 2.57864	Log = 3.22401	52.3' to 53.5' = 29 " " " " " " " " " " " "

Time
chilled
48 hours

TABLE X. *An experiment to ascertain if subculture during maximum growth is followed by a lag.**B. coli* Escherich. Medium = peptone water.

Times, mins	Culture A		Subculture B		Subculture C	
	No. per c.c.	No. of colonies counted	No. per c.c.	No. of colonies counted	No. per c.c.	No. of colonies counted
0	328	105	—	—	—	—
15	231	74	—	—	—	—
30	209	67	—	—	—	—
45	275	66	—	—	—	—
60	420	101	—	—	—	—
120	1900	76	—	—	—	—
122	—	—	479	115	—	—
135	—	—	835	167	—	—
150	—	—	1355	190	—	—
165	—	—	2010	201	—	—
180	—	—	3450	207	—	—
185	—	—	—	—	975	234
192	32,300	323	—	—	—	—
200	—	—	—	—	1788	322
215	—	—	—	—	2191	263
230	—	—	—	—	4425	354
245	—	—	—	—	5830	350
259	236,000	590	—	—	—	—
262	—	—	46,300	463	—	—
327	—	—	—	—	97,800	978
328	—	—	528,250	634	—	—
329	2,255,000	902	—	—	—	—
Time interval, mins.	Generation time, mins.	No. of generations	Generation time, mins.	No. of generations	Generation time, mins.	No. of generations
0 to 120	47	2·53	—	—	—	—
60 to 120	27	2·17	—	—	—	—
120 to 192	17	4·08	—	—	—	—
122 to 180	—	—	20	2·85	—	—
122 to 328	—	—	20·3	10·10	—	—
180 to 262	—	—	22	3·75	—	—
185 to 245	—	—	—	—	23	2·58
192 to 259	23	2·86	—	—	—	—
245 to 327	—	—	—	—	20	4·07
259 to 329	21	3·25	—	—	—	—
262 to 328	—	—	19	3·51	—	—

result had obtained and a slowing of growth equal to one minimum generation time had occurred, we would have found the generation time of the first hour in each experiment to be about 30 minutes, *i.e.* we would have had two generations during the first hour as against three in the second. All my experiments are incompatible with such a result.

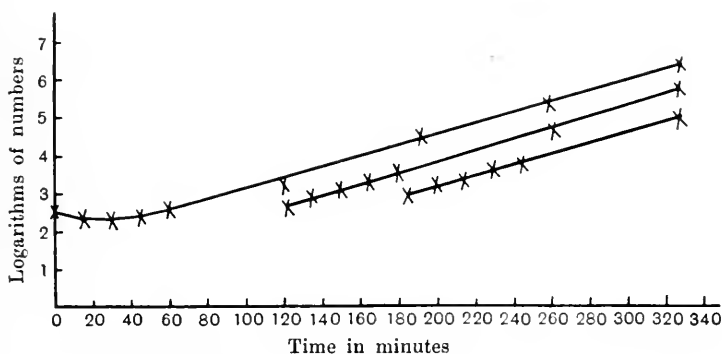


Chart 3 (with Table I). Subculture during maximum growth and lag.

Effect of temperature on lag.

Lane-Claypon (1909) states that the latent period with *B. coli* extended from one to six hours as the temperature fell from 42° to 20° C. During this fall of temperature the generation time lengthened four times. The published protocols of her experiments unfortunately do not deal with the lag period. It is obvious that lag and rate of growth are both affected by temperature but according to Lane-Claypon's unpublished figures not precisely equally. There do not appear to be many experimental data published on this subject. H. Chick (1913) however has shown that in serum the lag at temperature 40° C. is about one hour while at 20° C. it is 4½ hours. It is rather interesting to note that the generation time of *B. coli* in serum was affected very similarly.

Preliminary work suggests that the lag of *B. coli*, growing in peptone water at 20° C., is about six hours when inoculated from a parent culture of 15 hours. But as the experiments are only preliminary I propose to delay publication until I have more exact data on the subject.

Effect of age of parent culture on lag.

Max Müller (1895) demonstrated that an old parent culture was associated with a long lag in the subculture. This has been confirmed by Rahn (1906) and Coplans (1909).

I have repeated this work and have had no difficulty in observing a marked difference in the lags of subcultures made from cultures of *B. coli* grown at 37° C. for 17 hours and four days respectively. The parent cultures and subcultures were grown in each case at 37° C. It will be noticed on consulting the charts illustrative of this point that there appears to be no definite prolongation of the lag in passing from a parent culture of four days, to one of 12 days. Indeed, in the case recorded, it appears slightly less pronounced in the latter. In another experiment four day and eight day parent cultures gave the same lag on subculture. The fact that prolongation of lag is a marked feature in comparatively young cultures (of 17 hours to four days in the case of *B. coli* growing at 37° C.), while as the culture gets older no further prolongation occurs, is of considerable importance from the theoretical standpoint. This fact lends no support to the view that lag is an expression of injury. Table XI shows the details of one out of two experiments performed to elucidate this point. An endeavour was made in each experiment to obtain frequent observations at the critical periods.

The nature of the medium and its effect upon lag.

In subculturing from one medium to another it is common knowledge that long periods of lag in growth may occur, apparently depending on some adaptation.

If adaptation has been secured, however, by subculturing two series of the same organism, each series on a special medium, it may be found that the lag obtained on subculture on the respective media varies with the medium. In illustration of this point I may mention that Coplans has shown that subculture of a dulcitate peptone water culture of *B. coli* on to dulcitate peptone water gives a longer lag than a subculture of a peptone water culture on to peptone water, though the parent cultures are of the same age. I have repeated this experiment and have been able to confirm the result. See Table XII and Chart 4.

This effect of dulcitate in my experiment is not due to the large size of the inoculation in the case of the dulcitate culture, as an examination

TABLE XII. *Medium and Lag.*

A) The dulcete culture was 17 hours old, it was inoculated from a 15 hours' culture.
B) The peptone water culture was also 17 hours old. Each of these parent cultures was grown at 37°C. *source*

A' = 9 c.c. of dulcete broth inoculated with 6 drops of $\frac{1}{10000}$ dilution of the above dulcete culture.

A = 9 c.c. of culture broth inoculated with 0.1 c.c. of peptone water culture.	"	"	"
B' = 9 c.c. of peptone water	"	"	"
	"	$\frac{1}{100}$	"

Incubated at 37° C. The respective solutions were warmed to 37° C. before inoculation. 1 drop = 0.02 c.c.

		0	60 mins.	80 mins.	100 mins.	120 mins.	150 mins.	180 mins.	300 mins.	Generation times mins.
A.	A ₁	6 drops = 583	5 drops = 560	3 drops = 330	3 drops = 453	1 drop = 274	5 drops of $\frac{1}{10}$ dil. = 348	5 drops of $\frac{1}{30}$ dil. = 282	5 drops of $\frac{1}{100}$ dil. = 213	0 to 120 = 77
	A ₂	6 " = 631	5 " = 586	3 " = 412	3 " = 523	1 " = 321	5 drops ditto = 285	5 drops ditto = 270	5 drops ditto = 237	120 to 180 = 24
		Average per drop = 101 Log = 2.00432	Average per drop = 114 Log = 2.05690	Average per drop = 123 Log = 2.08991	Average per drop = 162 Log = 2.20952	Average per drop = 297 Log = 2.47276	Average per drop = 633 Log = 2.80140	Average per drop = 1650 Log = 3.21748	Average per drop = 45,000 Log = 4.65321	180 to 300 = 25
B.	B ₁	6 drops = 281	5 drops = 330	3 drops = 314	3 drops = 472	1 drop = 290	5 drops of $\frac{1}{10}$ dil. = 339	5 drops of $\frac{1}{30}$ dil. = 259	5 drops of $\frac{1}{100}$ dil. = 379	0 to 120 = 45
	B ₂	6 " = 282	5 " = 370	3 " = 327	3 " = 525	1 " = 310	5 drops ditto = 344	5 drops ditto = 261	5 drops ditto = 414	120 to 180 = 25
		Average per drop = 47 Log = 1.67210	Average per drop = 70 Log = 1.84510	Average per drop = 106 Log = 2.02531	Average per drop = 166 Log = 2.22011	Average per drop = 300 Log = 2.47712	Average per drop = 683 Log = 2.83442	Average per drop = 1560 Log = 3.19312	Average per drop = 79300 Log = 4.89927	180 to 300 = 21

of the figures on that subject shows that we are dealing with inoculations of such a size as to be but slightly affected by this factor. It will be noted, however, that the dulcitate parent culture had about 5.3 times the population of the peptone water parent culture and this may have rendered it similar to a peptone water culture of much greater age. Age of parent culture has already been shown to prolong lag.

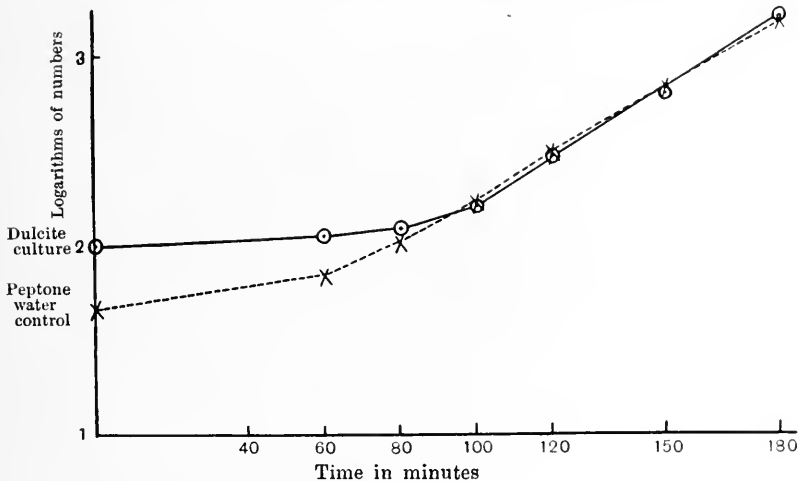


Chart 4. The nature of the medium and its effect upon lag.

Heat-stable bacterial products and lag.

It has been suggested that in the supernatant fluid of centrifuged cultures there are heat-stable bodies which may affect lag. Experiments performed to test this point gave no indication that any marked effect on lag resulted.

Table XIII and Chart 5 give the details of one such experiment from which it will be seen that these products have but little effect on lag, and apparently the slight effect they do exercise is in the direction of lengthening it.

The details of the experiment and technique employed appear in the table.

Inhibiting agents.

Eijkmann (1904) and others have long held the view that inhibition of growth in cultures is due to thermolabile inhibiting agents produced by the growth of the organisms, and it has naturally been thought that these bodies play an important part in the production of bacterial lag.

TABLE XIII. *Heat-stable Products.*

A = 1½ c.c. of overnight culture of <i>B. coli</i> in peptone water centrifuged to remove the organisms and heated 15 mins. at 100° C.	} Each of A, B, C, and D was inoculated with 1 drop of $\frac{1}{100}$ dilution of the original overnight culture of <i>B. coli</i> from which they themselves had been derived.
B = 1½ c.c. of overnight culture of <i>B. coli</i> in peptone water heated as above but without previous centrifuging.	
C = 1½ c.c. of overnight culture of <i>B. coli</i> in peptone water centrifuged after heating for 15 mins. at 100° C.	
D = 1½ c.c. of ordinary peptone water as control.	

1 drop = 0.02 c.c.

	0	120 mins.	240 mins.	360 mins.	Generation times, mins.
A.	4 drops = 183 Average per drop = 46 Log = 1.66276	4 drops = 484 Average per drop = 121 Log = 2.08279	6 drops of $\frac{1}{10}$ dil. = 1003 Average per drop neat = 1670 Log = 3.22272	3 drops of $\frac{1}{100}$ dil. = 674 Average per drop neat = 22466 Log = 4.35141	0 to 120 = 86 120 to 240 = 32 240 to 360 = 32
B.	4 drops = 179 Average per drop = 45 Log = 1.65321	4 drops = 538 Average per drop = 135 Log = 2.13033	1 drop of $\frac{1}{10}$ dil. = 218 Average per drop neat = 2180 Log = 3.33846	1 drop of $\frac{1}{100}$ dil. = 270 Average per drop neat = 27000 Log = 4.43136	0 to 120 = 76 120 to 240 = 30 240 to 360 = 33
C.	4 drops = 152 Average per drop = 38 Log = 1.57978	4 drops = 455 Average per drop = 114 Log = 2.05690	1 drop of $\frac{1}{10}$ dil. = 181 Average per drop neat = 1810 Log = 3.25768	3 drops of $\frac{1}{100}$ dil. = 739 Average per drop neat = 24600 Log = 4.39094	0 to 120 = 76 120 to 240 = 30 240 to 360 = 32
D.	4 drops = 150 Average per drop neat = 38 Log = 1.57978	4 drops = 469 Average per drop = 117 Log = 2.06819	9 drops of $\frac{1}{100}$ dil. = 1222 Average per drop neat = 6800 Log = 3.83251	1 drop of $\frac{1}{1000}$ dil. = 380 Average per drop neat = 190000 Log = 5.27875	0 to 120 = 74 120 to 240 = 20 240 to 360 = 25

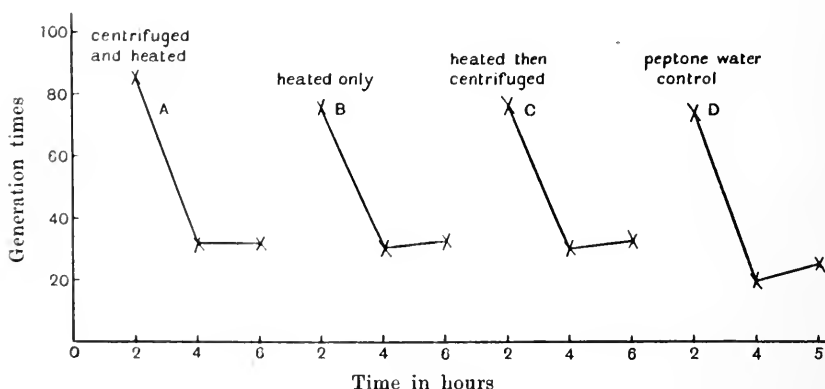


Chart 5. Heat-stable bacterial products and lag.

The existence of these bodies and the part they play in bacterial inhibition have been the subject of warm controversy. Without coming to any decision with regard to these agents I made repeated attempts, by washing in saline and Ringer's fluid for one or two hours, to obtain seeding material from 17 hours' peptone water cultures which would be accompanied by no lag on subculture. I found that this method of treatment did not remove the lag. On the other hand after washing with saline the lag was slightly increased, while in the case of Ringer two hours immersion prevented subsequent growth. The proper salt ratio of a fluid with which to wash *B. coli* without injury to it, has probably not been found, so that in this experiment one may be simply substituting injury for the ordinary cause of lag. I do not propose to give the details of these experiments, the interest of which would have lain in the finding of a positive result.

TABLE XIV. EXP. A. *Supernatant and Lag.*

B. coli in autoclaved peptone water (1 % + 5 % salt), grown overnight (24 hours) at 37° C. The organisms were centrifuged off and the supernatant plated and found to contain 148 organisms in 1 drop of $\frac{1}{10}$ dilution. This stood in cold room at 0° C. overnight. It was plated out next day and gave the numbers given below at time 0. It was then warmed and placed in the incubator at 37° C. and counted as indicated. A culture of *B. coli* in peptone water has after 20 hours growth a generation time varying from approximately six hours upwards. 1 drop = 0.02 c.c.

	0	120 mins.	360 mins.	Generation times, mins.
A.	1 drop of $\frac{1}{10}$ dil. = 156	1 drop of $\frac{1}{40}$ dil. = 89	1 drop of $\frac{1}{5000}$ dil. = 19	0 to 120 = 103
	1 " " = 168	1 " " = 105	1 " " = 24	
	1 " " = 166	3 drops " = 256	5 drops " = 109	120 to 360 = 47
	1 " " = 163	3 " " = 281	5 " " = 138	
	Average per drop neat = 1630	Average per drop neat = 3650	Average per drop neat = 120830	
	Log = 3.21219	Log = 3.56229	Log = 5.08207	

EXP. B. *Supernatant and Lag.*

B. coli was grown on peptone water (1 % peptone + 5 % salt) for 4 days at 37° C., then centrifuged and placed in the cold room overnight. The rest of this experiment was conducted as A and gave the following result.

	0	120 mins.	495 mins.	Generation times, mins.
B.	1 drop of $\frac{1}{10}$ dil. = 14	1 drop of $\frac{1}{10}$ dil. = 17	1 drop of $\frac{1}{1000}$ dil. = 85	0 to 120 = 113
	5 drops " = 72	1 " " = 30	1 " " = 70	
		5 drops " = 151	10 drops " = 666	120 to 495 = 47
		5 " " = 160	10 " " = 806	
	Average per drop neat = 143	Average per drop neat = 298	Average per drop neat = 73950	
	Log = 2.15534	Log = 2.47422	Log = 4.86894	

Growth on supernatant and lag.

If a peptone water culture of *B. coli* be centrifuged it is found that the few bacteria remaining in the supernatant commence to grow again at a quick rate but not without a period of lag. I have not submitted full quantitative data dealing with this fact because those I have obtained so far are of a preliminary character. In Table XIV are given the data of two such experiments, each done in duplicate, from which it is seen that a marked lag occurred in each case. The lag was of so pronounced a character that it probably could not be accounted for by the fact that the culture had been overnight in the cold room. Further, after two hours, the growth of the residual organisms becomes so rapid as to suggest that no very powerful inhibiting agents of any kind are present.

SUMMARY OF RESULTS.

(1) If *B. coli* be subcultured into another sample of the same medium when growing at full pace, it will continue to grow at the same pace.

(2) If the maximum rate of growth be interrupted by a short application of cold, growth will recommence without lag on the temperature being raised. If the cold be long continued, lag will tend to reappear.

(3) Differences in the size of inoculum have practically no effect on lag in the case of large inoculums, in the case of small ones, on the other hand, diminution of the seeding has the effect of lengthening lag, and this lengthening effect is more marked the smaller the seedings become.

(4) Lowering the temperature lengthens the lag. The effect is very similar to the effect on growth.

(5) The older a parent culture (within limits) the longer the lag.

(6) The length of lag varies with the medium even if adaptation has been arranged for beforehand.

(7) Heat-stable products in *B. coli* cultures on peptone water have, in the case of overnight cultures, but little effect on lag.

(8) After washing the bacteria for two hours with saline in order to remove possible inhibiting agents, it was found that the lag, on subculture, still occurred and was indeed slightly longer.

(9) If a peptone water culture of *B. coli* be centrifuged, it is found that the few bacteria remaining in the supernatant commence to grow again at a quick rate but not without a period of lag.

Discussion of modern views on the subject.

It has been suggested that :

(a) Something must be secreted into the medium before maximum growth occurs. Against this is the fact that subculture when growth is maximal is followed by maximal growth. On the other hand this factor may come in in the case of subcultures of slowly growing parent cultures ; it might account for the fact that increase of size of inoculum tends to diminish lag.

(b) Any change from one medium to another requires adaptation and this is attended with initial slow growth.

This is not the explanation of any of the lags with which we have been dealing as no such change occurred in any case.

(c) The osmotic pressure of the parent culture medium is different from that of the new sample of the same medium. A marked lag, as we have seen, is however present in the case of the residual organisms left after centrifuging a 24 hours' culture of *B. coli*. This may be partly accounted for by the exposure to cold of the culture after centrifuging, but a reference to previous experiments detailed in this paper shows that this is quite insufficient to account for the total lag, and equally it cannot be explained on variations of osmotic pressure of the medium.

(d) The presence in the medium of the end products of metabolism are essential to maximal growth. It has been shown that emulsin splits salicin better in the presence of saliginin and glucose. This cannot have a general application however in the case of bacterial lag for the same reason as negatives hypothesis (a).

(e) The transferred organism may not be viable, some of them may die ; since, however, all our initial populations are counted by their power to grow in agar plates, this factor probably does not come in.

(f) The transferred organisms may agglutinate. This factor comes in to some extent in dealing with serum and milk as culture media, but I was unable to obtain any evidence of it in the case of *B. coli* growing on peptone water.

(g) That the organisms are injured by the accumulated metabolic products of the parent culture. In the case of *B. coli* growing on peptone water, however, this does not appear to be the complete explanation, since the residual organisms after the culture is centrifuged are able to attain a generation time of 47 minutes though the whole culture had a generation time of about six hours.

(*h*) That the inoculum consists of organisms having individually different powers of growth and that during the lag the selection of a quick growing strain occurs in response to some selecting agent in the peptone. This would present an analogy to the selection of bacteria which goes on in media containing a fermentable sugar.

Under this scheme a subculture made during the lag would show a lag somewhat shorter than the lag of the parent culture, while a subculture made during the so-called logarithmic period would show no lag. This as a matter of fact is what happens. At the end of the logarithmic period a selection would again take place in response to some other constituent of the culture and so conditions would result which would entail lag on subculture. Much may be said for this view, reasoning by analogy, but if it were true one would naturally suppose if two subcultures were made from the same parent culture marked differences in the rates of growth might occur between them, especially if the seedings were small. Now a reference to the experiments on size of inoculation and lag will show that the duplicate tubes always grew about the same rate and the differences between the members of pairs with small seedings were no greater than between the members of pairs with large seedings. I have obtained the same result in many unpublished experiments. This does not favour the existence of great variability in power of growth in the population.

(*i*) In addition to these various views it appeared desirable to exclude the possibility of inertia on the part of the bacteria, and for this purpose the chilling experiments were done. These experiments show that stoppage of growth by cold does not of necessity occasion a lag before subsequent maximal growth is attained.

(*j*) It has been shown that an induction period occurs in certain chemical reactions and in some instances at least this has been shown to be due to the fact that the reaction takes place in stages. It is quite obvious that if substance *C* is produced not from substance *A* directly, but from an intermediate substance *B*, then the maximum production of *C* will occur, not at the commencement of the reaction when none of *B* is present but only after *B* has reached maximum concentration. It seems possible that in bacterial lag we have a phenomenon that may be explained on this purely chemical basis. I venture to suggest:

(1) Some of the constituents of the bacterial protoplasm are probably synthesized in steps, perhaps by a succession of enzymes.

(2) Maximum growth presupposes the optimum concentration

attainable by the bacterium, of the intermediate bodies in the steps of the syntheses.

(3) When bacteria have stopped growing these intermediate bodies tend to diffuse out into the medium or disappear in some other way and their concentration in the bacteria falls.

(4) That transfer to a new medium is only followed by maximal growth when these intermediate products have again attained optimum concentration in the organism.

This view of lag would be in accord with the known facts. It might also account for that portion of the lag which can be removed by increasing the inoculum and which may depend on the absence of intermediate products in the medium. Since, however, no lag occurs if an organism be transferred while growing at maximum rate, we must look upon their presence in the medium as being of secondary importance. This absence of lag under these conditions would appear to be due to the fact that the intermediate bodies are present in the transferred organisms in optimum concentration. The effect of the cold may be explained on this hypothesis in this way—if the cold be evanescent these bodies have not time to disappear by diffusion or otherwise, if it be long continued they have, and on that account lag tends to reappear.

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MATHEMATICAL ANALYSIS OF THE LAG-PHASE IN BACTERIAL GROWTH.

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(With 9 Charts and 1 Text-figure.)

OF the large number of experiments recorded by Penfold (1914) in connexion with the question of lag in bacterial growth, a certain proportion have been carried out in such detail as to render them eminently suitable for mathematical analysis. In all work of this kind, which is intended to throw light on the numerical aspect of bacterial growth, it is desirable to take observations at frequent and, if possible, regular intervals, during the period of lag, and to count a sufficiently large number of colonies on the plates. To secure this last and most important desideratum, orientating experiments must be performed, from the results of which one is enabled to calculate what dilution of the culture at any given period of growth will yield a tolerably large and accurately countable plate population.

The experiments which Penfold has performed on the influence exerted on lag by variations in the initial seeding, lend themselves admirably to mathematical analysis, and we propose here to confine our mathematical treatment solely to the two parallel series of experiments dealing with this point. The literature of the subject has been fully dealt with in the paper by Penfold (*loc. cit.*).

The term "lag" has been used in different senses by different writers on the subject. By some, it is understood as a definite period

during which growth is apparently in abeyance. More usually, it is taken to mean that period which elapses between the time of seeding and the point at which the velocity of reproduction attains its maximal level or, in other words, the point at which the generation-time becomes minimal.

The attainment of this minimal level of generation-time is the prelude to what is known as the second or logarithmic phase of bacterial growth during which the generation-time remains at this constant minimum.

In most recorded work on the multiplication of bacteria, analysis of the lag-phase has received scanty attention, whereas the second or logarithmic phase has been very fully dealt with. There can be no doubt, however, that the phenomenon of lag is of profound significance in the life-history of the organism, and in the following analysis we hope to show that this phase proceeds in an orderly fashion and according to a perfectly definite law, until the logarithmic phase commences. In other words, we shall hope to show that the generation-time diminishes steadily and regularly from the commencement of seeding till a minimal length is reached.

What the minimal generation-time is to which a *B. coli* culture can attain, is difficult to state with exactness, but, in practice, it has not been possible to demonstrate a generation-time of less than 18-20 minutes. That the interval between two successive divisions cannot be reduced below a certain minimum, is an important fact in the mechanics of bacterial growth, and has to be reckoned with in any theory which attempts to explain growth in a nutrient medium as a continuous or discontinuous process. This point will be discussed later. At present, there are no data covering the whole period of growth from the commencement of seeding to the period when growth ceases altogether and cell-death comes into play. Ample observations are available on the logarithmic phase or phase of constant generation-time, but only a few isolated observations have been made on the subsequent phase during which the generation-time increases till it probably again becomes infinite as at the commencement of seeding. Accurate and sufficient data on these phases, as also on that of cell-death, will, it is hoped, soon be forthcoming.

*Analysis of experiments on lag.**Series A. Experiments 1—4.*

In these four experiments, the initial seedings, calculated from the plates, were respectively 217·5, 59·4, 20·2, and 2·4 bacilli per drop, or roughly as 100:25:10:1.

Incubation at 37° C.

Experiment 1.

Time (X)	Bacilli (Y)	Log X	Log Y
0	217·5	—	2·3374
45	287	1·653	2·4578
60	345	1·778	2·5378
80	470	1·903	2·6721
100	718	2	2·8561
120	1362	2·079	3·1341
150	2535	2·176	3·4039
180	7610	2·255	3·8813

Transfer the origin from (0, 0) to (0, 2·3374).

Then log Y or Y' becomes

0
0·1204
0·2004
0·3347
0·5187
0·7967
1·0665
1·5439

The values of log X and of log Y were plotted out in the usual way and from the shape of the smooth curve so obtained, it was conjectured that an equation of the form $X^n = kY'$ would fit most closely the observed data. If this proved to be the case, then the points obtained by plotting log X against log Y', *i.e.* against log (log Y) ought to lie on a straight line.

The values of log (log Y) are as follows :

— ∞
1·0806
1·3016
1·5246
1·7149
1·9012
0·0277
0·1886

These values were then plotted against the corresponding values of log X and it was found that the points so obtained lay very closely along a straight line the tangent of whose inclination to the axis of X was 1·88.

This value of *n*, viz. 1·88, gives the following values for log *k* the other constant in the equation $X^n = k \log Y$.

4·027
4·039
4·053
4·045
4·007
4·063
4·050

These values indicate a quite satisfactory constancy of k .

The average value of k is found to be 10988.

Transfer back to the old origin and we find that the following equation

$$X^{1.88} = 10988 \log \frac{Y}{217.5}$$

will fit most closely the experimental data.

Experiments 2, 3, and 4 were treated similarly and the following values for the exponent n were obtained, 1.77, 1.56 and 1.56 respectively.

These values for n give the following values for $\log k'$, $\log k''$ and $\log k'''$ resp.

Exp. 2	Exp. 3	Exp. 4
$n=1.77$	$n=1.56$	$n=1.56$
Log k'	Log k''	Log k'''
3.814	3.343	3.364
3.750	3.391	3.337
3.795	3.382	3.402
3.837	3.371	3.447
3.790	3.367	3.433
3.801	3.365	3.387
3.811	3.346	3.358

The average values for k' , k'' , and k''' are 6322, 2329, and 2465 resp.

The following equations, therefore, will fit most closely the experimental data in Experiments 2, 3, and 4.

$$X^{1.77} = 6322 \log \frac{Y}{59.4},$$

$$X^{1.56} = 2329 \log \frac{Y}{20.2},$$

$$X^{1.56} = 2465 \log \frac{Y}{2.4},$$

Series B. Experiments 5—8.

In this parallel series, the initial seedings were 144, 35.7, 14, and 1.7 bacilli per drop resp. or roughly as 100:25:10:1.

The data from these experiments were dealt with in a similar way and the following values for the exponent n were obtained, viz. 1.97, 1.74, 2.01 and 2.7 resp.

These values for n gave the following values for $\log k$, $\log k'$, $\log k''$, and $\log k'''$ resp.

Exp. 5	Exp. 6	Exp. 7	Exp. 8
$n=1.97$	$n=1.74$	$n=2.01$	$n=2.7$
Log k	Log k'	Log k''	Log k'''
4.298	3.565	4.667	6.225
4.228	3.762	4.366	6.160
4.184	3.723	4.374	6.052
4.185	3.730	4.351	5.985
4.178	3.744	4.343	6.017
4.239	3.736	4.359	6.025
4.236	3.742	4.379	6.020

It will be observed that the constancy obtained for $\log k$, $\log k'$, $\log k''$, and $\log k'''$ in these four experiments is very satisfactory. The following equations will, therefore, fit very closely the experimental data.

Bacterial Lag

$$X^{1.97} = 16732 \log \frac{Y}{144},$$

$$X^{1.74} = 5483 \log \frac{Y}{35.7},$$

$$X^{2.01} = 23020 \log \frac{Y}{14},$$

$$X^{2.7} = 1045000 \log \frac{Y}{1.7}.$$

In the following tables the observed values for Y (Bacilli per drop) are compared in parallel columns with those calculated from the theoretical equations.

Series A.

Exp. 1.

$$\text{Equation: } X^{1.88} = 10988 \log \frac{Y}{217.5}.$$

X (time)	Y (observed)	Y (theoretical)
0	217.5	217.5
45	287	284.49
60	345	344.95
80	470	480.67
100	718	726.88
120	1362	1188.2
150	2535	2888.4
180	7610	8288.9

Exp. 2.

$$\text{Equation: } X^{1.77} = 6322 \log \frac{Y}{59.4}.$$

X (time)	Y (observed)	Y (theoretical)
0	59.4	59.4
45	80	80.72
60	105.5	99.01
80	140.5	138.99
100	189.5	210.03
120	354	339.4
150	789	790.6
180	1950	2112.8

Exp. 3.

$$\text{Equation: } X^{1.56} = 2329 \log \frac{Y}{20.2}.$$

X (time)	Y (observed)	Y (theoretical)
0	20.2	20.2
45	30	29.37
60	35.2	36.33
80	49.1	50.64
100	73.8	74.37
120	114.4	113.86
150	240	234.72
180	621	525.40

Exp. 4.

Equation: $X^{1.56} = 2465 \log \frac{Y}{2.4}$.

X (time)	Y (observed)	Y (theoretical)
0	2.4	2.4
45	3.5	3.42
60	4.5	4.18
80	5.6	5.72
100	7.1	8.22
120	10.6	12.31
150	25	24.35
180	67	52.03

Series B.

Exp. 5.

Equation: $X^{1.97} = 16732 \log \frac{Y}{144}$.

X (time)	Y (observed)	Y (theoretical)
0	144	144
40	170	175.39
60	222	223.2
80	335	311.61
100	533	477.5
120	966	800.49
150	1885	2067.84
180	5840	6537.6

Exp. 6.

Equation: $X^{1.74} = 5483 \log \frac{Y}{35.7}$.

X (time)	Y (observed)	Y (theoretical)
0	35.7	35.7
40	52.4	46.19
60	58.5	60.11
80	87	84.50
100	130	126.84
120	199	203.73
150	472	465.52
180	1194	1215.22

Exp. 7.

Equation: $X^{2.01} = 23020 \log \frac{Y}{14}$.

X (time)	Y (observed)	Y (theoretical)
0	14	14
40	15.2	16.52
60	20.3	20.37
80	26.8	27.31
100	41	39.91
120	67.8	63.18
150	151.3	148.96
180	371	422.80

Bacterial Lag

Exp. 8.

$$\text{Equation: } X^{2.7} = 1045000 \log \frac{Y}{1.7}.$$

X (time)	Y (observed)	Y (theoretical)
0	1.7	1.7
40	1.75	1.78
60	1.88	1.95
80	2.25	2.30
100	3.09	2.95
120	4.22	4.19
150	8.66	8.87
180	25.16	25.43

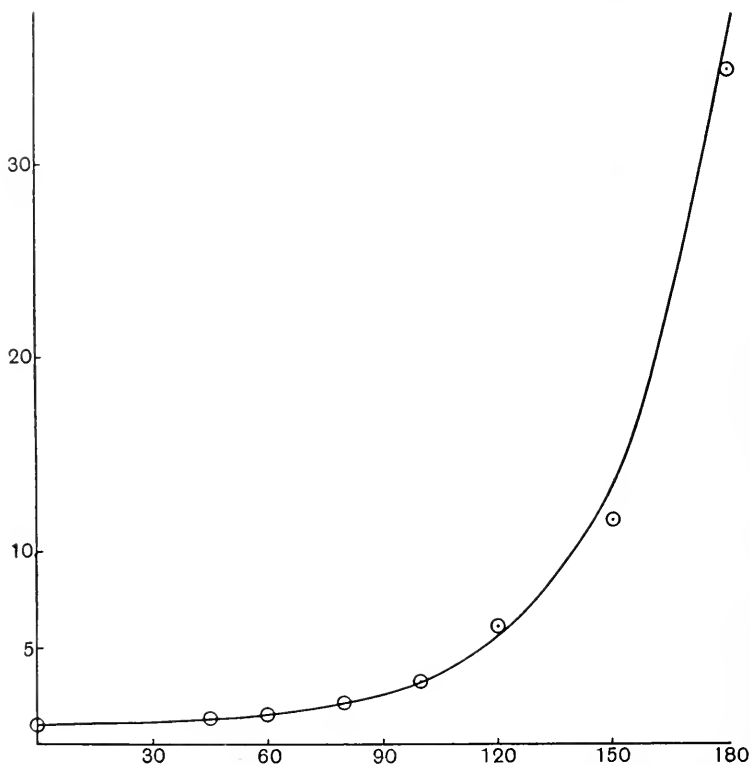


Chart I. Series A. Exp. 1. Ordinates. Bacilli. (Initial seeding taken as 1.)
Abcissae. Time (minutes).

In Charts I—VIII the theoretical curves have been drawn to scale and the observed points have been inserted as circles. The closeness of fit is, in most cases, very satisfactory.

Summarising the results of these eight experiments, we find that during the whole lag-phase, growth takes place in a perfectly regular

fashion, the bacterial content (Y) at any time (X) being very satisfactorily determined from the general equation

$$(X^n = k \log Y/s),$$

where n and k are constants and s is the initial seeding.

The value of the exponent n falls slightly as the seeding is reduced except in the last two experiments of Series B.

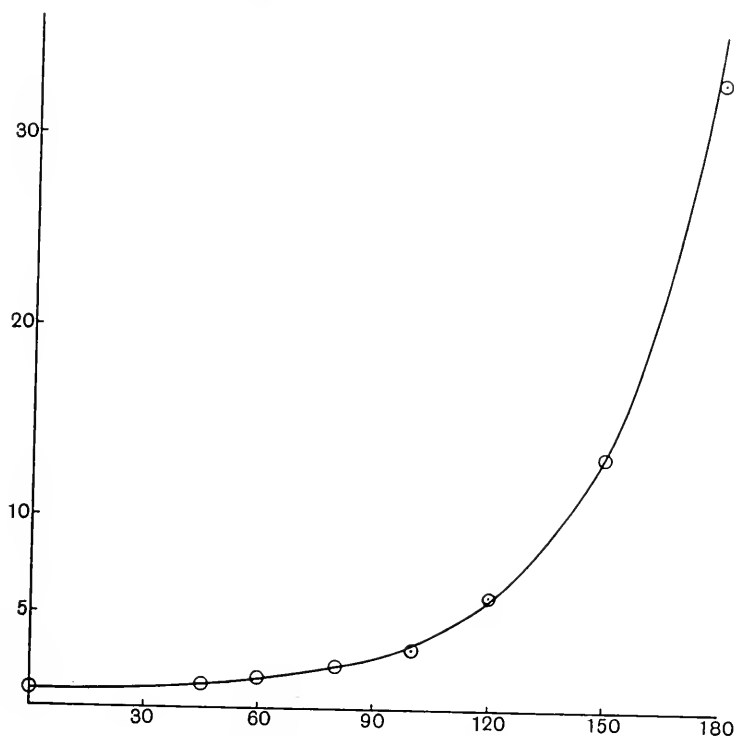


Chart II. Series A. Exp. 2.

The figures may be conveniently tabulated here :

Seeding	Series A	Series B
100	1.88	1.97
25	1.77	1.74
10	1.56	2.01
1	1.56	2.7

The two discrepant values for n in Series B will receive more full consideration when we come to discuss the question of generation-time during lag, but it would appear that in the case of very small

initial seedings (as in Experiments 7 and 8 of Series B) where the number of bacilli per drop were only 14, and 1.7 respectively, the latter part of the lag-phase may be characterised by a very marked acceleration of the growth-rate. This will appear when the generation-times have been calculated.

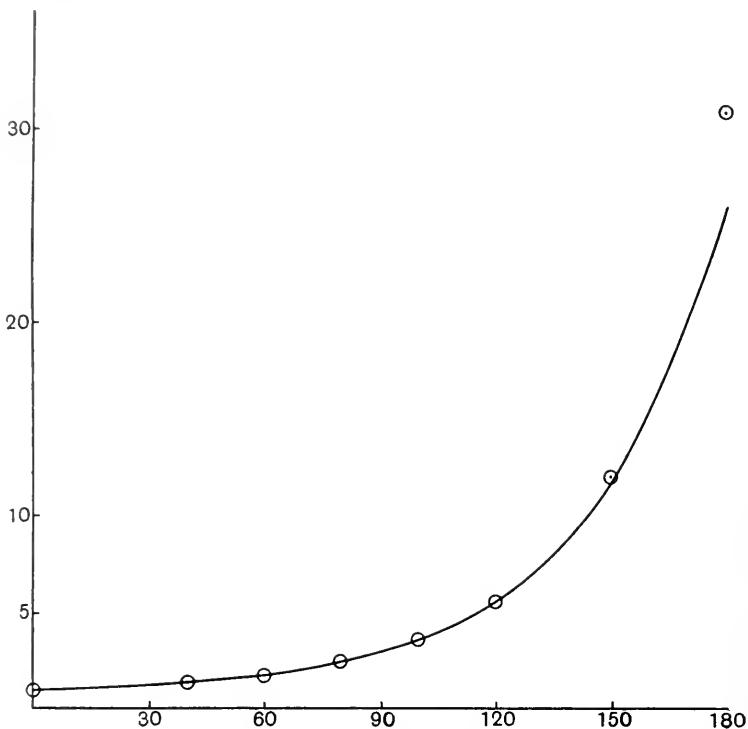


Chart III. Series A. Exp. 3.

The question of generation-time.

The generation-time or the period which elapses between two successive divisions of an organism is usually calculated from the bacterial contents at the beginning and end of a certain arbitrary period. It represents essentially the mean generation-time during the period in question. If short and equal periods are chosen, a very fair impression can be obtained of the changes which this function undergoes in the course of growth. In practice, however, this is not always feasible but it is always possible to get an approximation to the value of the generation-time at any point, by determining the

inclination of the tangent to the log-curve at that point. From the mathematical expressions which we have obtained for the course of the lag-phase in the above series of experiments, it is possible to calculate with exactness the generation-time at any point in the course of the lag.

Let P be any point (x, y) on the curve and P' and P'' two points on either side of P whose co-ordinates are $(x - \delta x, y - \delta y)$ and $(x + \delta x, y + \delta y)$ respectively, where δx and δy are very small. If r = the number of generations between the bacterial contents corresponding to $y + \delta y$ and $y - \delta y$, we must have $r \log 2 = \log (y + \delta y) - \log (y - \delta y)$.

The whole period is $2\delta x$.

Therefore one generation-time equals $\frac{2\delta x \log 2}{\log (y + \delta y) - \log (y - \delta y)}$ *.

Let $y' = \log y$, then $dy' = \frac{1}{y} dy$.

The expression for the generation-time becomes

$$\frac{2\delta x \log 2}{y' + \delta y' - y' + \delta y'} = \frac{\delta x \log 2}{\delta y'}.$$

In the limit, when δx and δy are infinitely small, the value for the generation-time becomes $y \log 2 \frac{dx}{dy}$. Now $\frac{dx}{dy}$ can be obtained directly from the equation $x^n = k \log y/s$. We have $nx^{n-1} \frac{dx}{dy} = \frac{k}{y}$. Therefore the expression for the generation-time at the point P becomes

$$\frac{ky \log 2}{nyx^{n-1}} = \frac{k \log 2}{nx^{n-1}}.$$

* The expression $\frac{2\delta x \log 2}{\log (y + \delta y) - \log (y - \delta y)}$ may be otherwise reduced thus:

$$\log (y + \delta y) \text{ [expanded by Taylor's Theorem]} = \log y + \frac{\delta y}{y} - \frac{1}{2} \frac{\delta y^2}{y^2} + \frac{1}{3} \frac{\delta y^3}{y^3} - \&c.,$$

$$\log (y - \delta y) \text{ [do. do.]} = \log y - \frac{\delta y}{y} - \frac{1}{2} \frac{\delta y^2}{y^2} - \frac{1}{3} \frac{\delta y^3}{y^3} \&c.,$$

$$\therefore \log (y + \delta y) - \log (y - \delta y) = 2 \frac{\delta y}{y} + \frac{2}{3} \frac{\delta y^3}{y^3} \&c.$$

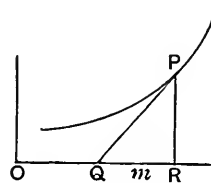
The expression for G. T. then becomes

$$\frac{2\delta x \log 2}{2 \frac{\delta y}{y} + \frac{2}{3} \frac{\delta y^3}{y^3} \&c.}.$$

In the limit, powers of δy may be neglected and G. T. becomes

$$y \log 2 \frac{dx}{dy} \text{ (as before).}$$

If the tangent at the point P cuts the axis of X at Q and the ordinate of P at R , then if the intercept between these two points be denoted by m , the G. T. at the point P is equal to $m \log 2$.



We can therefore determine the generation-time at any moment (x) after the commencement of growth, by substituting in this expression the value of x and the values determined for k and n .

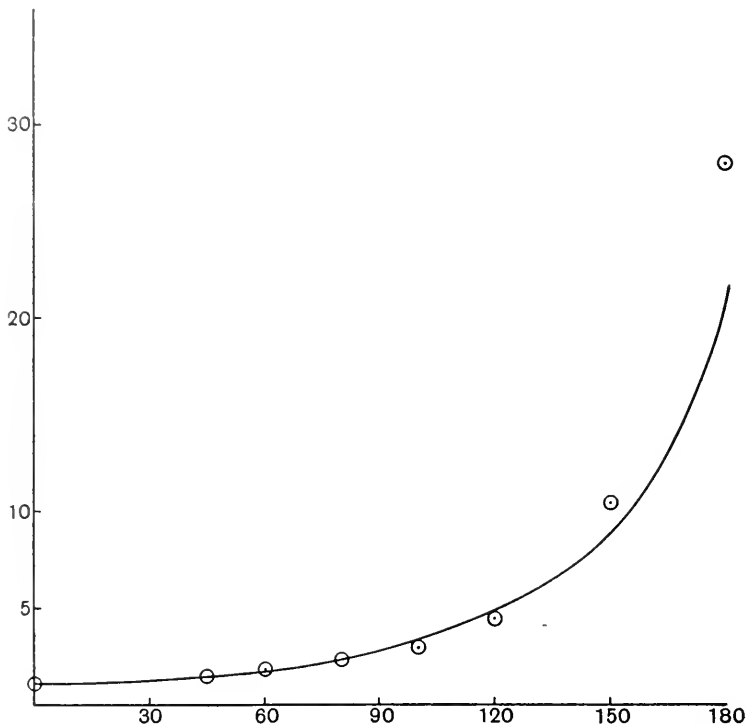


Chart IV. Series A. Exp. 4.

This determination has been made at half-hourly periods during the lag-phase of each of the eight experiments with the following results :

Series A.

	Exp. 1	Exp. 2	Exp. 3	Exp. 4
Time	G. T.	G. T.	G. T.	G. T.
30	88.19	78.36	66.90	70.81
60	47.92	45.95	45.38	48.03
90	33.54	33.62	36.16	38.27
120	26.04	26.94	30.78	32.58
150	21.40	22.69	27.17	28.75
180	18.23	19.72	24.53	25.96

Series B.

Time	Exp. 5 G. T.	Exp. 6 G. T.	Exp. 7 G. T.	Exp. 8 G. T.
30	103.4	76.5	111.0	359.1
60	48.14	45.82	55.09	110.5
90	32.55	33.97	36.56	55.47
120	24.63	27.48	27.35	33.97
150	19.80	23.29	21.88	23.29
180	16.62	20.33	18.20	17.07

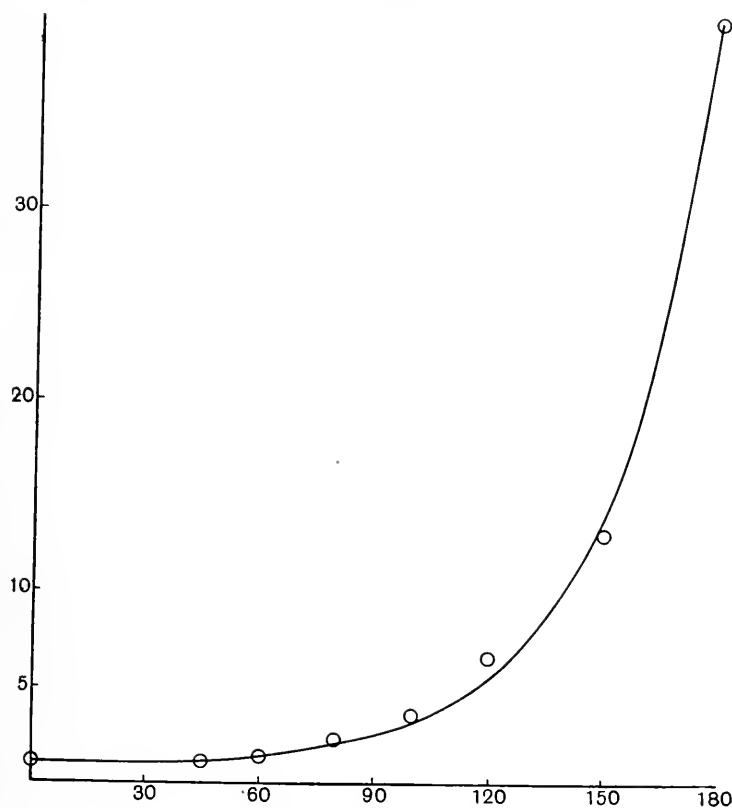


Chart V. Series B. Exp. 5.

Analysis of Series A. (Generation Times.)

In Exps. 1 and 2, in which the seedings were 217.5 and 59.4 bacilli per drop respectively, the generation-times attained at each half-hourly period are practically identical and at the end of the 3-hour period the minimum generation-time of 18-19 minutes has been reached.

The influence of reduction in the initial seeding is however very apparent in Exps. 3 and 4. Here the seedings were respectively 20.2 and 2.4 bacilli per drop. The value of the exponent n was in both cases 1.56 and consequently there is practically no difference between the generation-times attained at the half-hourly periods. In both experiments, however, the end of the lag period has not been reached in three hours, the final recorded generation-time being 24-25 minutes as compared with 18-19 minutes in Exps. 1 and 2.

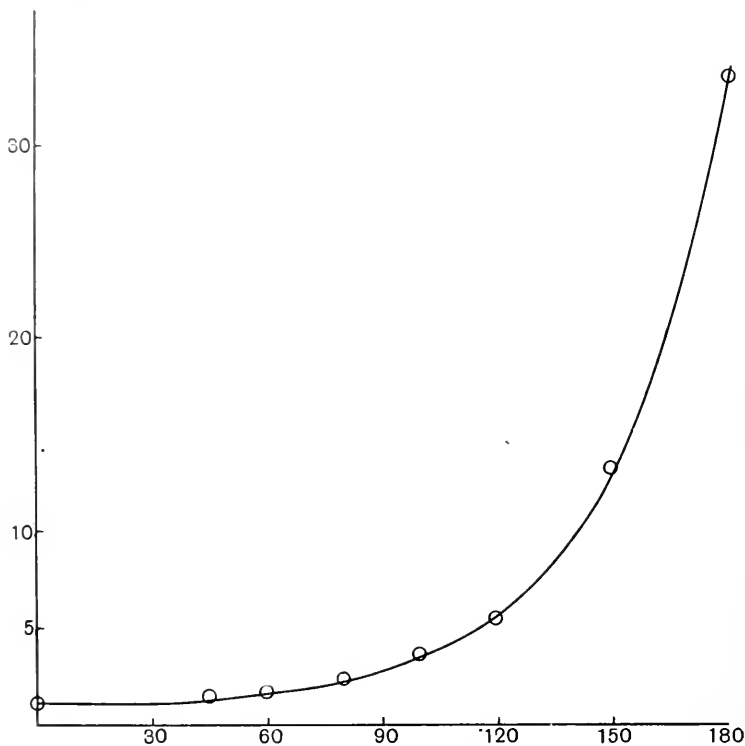


Chart VI. Series B. Exp. 6.

At each half-hourly period (after the 1st hour) the generation-times are distinctly longer by 3-6 minutes than the corresponding times in Exps. 1 and 2.

Series B.

In this series all have reached the minimum generation-time in three hours so that the total period of lag is practically the same in all cases. It will be observed, however, that at 90 minutes the generation-time of

Exp. 7 is appreciably behind those of Exps. 5 and 6 while that of Exp. 8 is very far behind. At 120 minutes the generation-time of Exp. 8 is still behind the others, but at the end of the next half-hour (at 150 minutes) the generation-time of Exp. 8 has reached the level of the others. The remarkable acceleration in rate of growth which has occurred in Exps. 7 and 8, during the latter period of the lag is of course simply an expression of the enhanced value of the

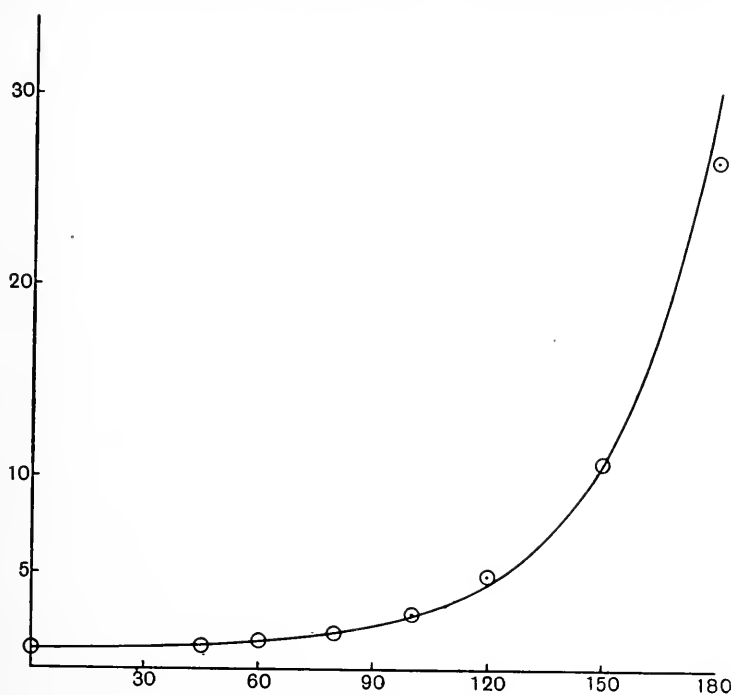


Chart VII. Series B. Exp. 7.

exponent n in the derived equations and at present we have no satisfactory explanation to offer for this fact. It may possibly find an explanation when the factors that enter into the causation of lag are more thoroughly understood.

The later phases of Growth.

It is not our purpose here to discuss in any detail the later phases of growth succeeding the lag. The second or logarithmic phase has been very thoroughly investigated by Lane-Claypon (1909) and others who find that during this phase the generation-time remains at a constant minimum.

Time and bacilli are related to each other by the simple equation $X = k \log Y$ so that, when the logs of the bacilli are plotted against time, the points so obtained lie on straight lines. The exponent of X is unity during this phase. Its duration varies with the medium in which the organisms are growing and with the temperature. In peptone-water, at 37° C., it lasts about three to six hours according to the size of seeding after which time the 3rd phase or phase of rising (*i.e.* slower) generation-time ensues. This phase proceeds till growth ceases and cell-death begins. Whether a plateau occurs during which the bacillary content remains at a constant maximum for a certain

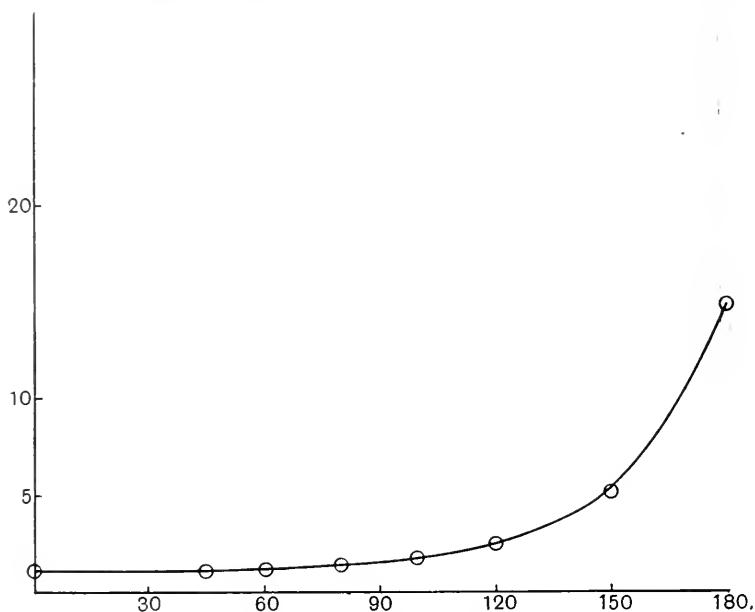


Chart VIII. Series B. Exp. 8.

time is not definitely established though there is some evidence in support of this occurrence. No complete data from a single experiment are available from the period of seeding onwards to the commencement of cell-death, but by a fortunate circumstance we are in a position to state the most probable course taken by the growth curve over a period including the lag, the 2nd phase, and a large portion of the 3rd phase.

An experiment was performed in which the initial seeding was 37.3 bacilli per drop and the first observation was made at the 3rd hour and at hourly intervals thereafter.

The figures obtained were as follows :

Time, hrs.	Bacilli per drop
0	37·3
3	1,692·5
4	7,833·3
5	46,000
6	231,000
7	553,888
8	974,166
9	1,166,666
10	1,643,333
11	2,112,222
12	2,396,470

Now, it will be noted that in Exp. 6 (Series B) the initial seeding was 35·7 bacilli per drop and the figure reached at three hours was 1194. We can therefore legitimately employ the figures for the lag-phase of Exp. 6 to complete the data in this new experiment where the lag-phase was not observed. From the equation for Exp. 6 it was calculated that a figure of 1692 bacilli per drop would have been reached in 186 minutes. The logarithmic phase of the new experiment lasted about 3 hours at a constant generation-time of about 25 minutes. At the following times therefore (increments of 25 minutes) the following figures would be reached :

Time, mins.	Bacilli per drop
186	1,692
211	3,384
236	6,768
261	13,536
286	27,072
311	54,144
336	108,288
361	216,576 (Actual figure obtained at
421	553,888 360 mins. was 231,000.)
481	974,166
601	1,643,333
661	2,112,222
721	2,396,470

The above figures and times along with those for the lag-phase of Exp. 6 have been plotted in Chart IX.

The actual course of the growth curve is indicated by the successive divisions *OA* (lag), *AB* (logarithmic phase), *BC* (3rd phase during which the curve passes through a point of inflexion and becomes concave to the axis of *X*). On the scale employed in Chart IX it is possible to exhibit the lag-phase *OA*, as a straight line only.

Had growth continued according to the law maintained during the lag, the growth curve would have followed the route AA' .

Had the logarithmic phase continued, the curve would have followed the route BB' instead of the actual route BC .

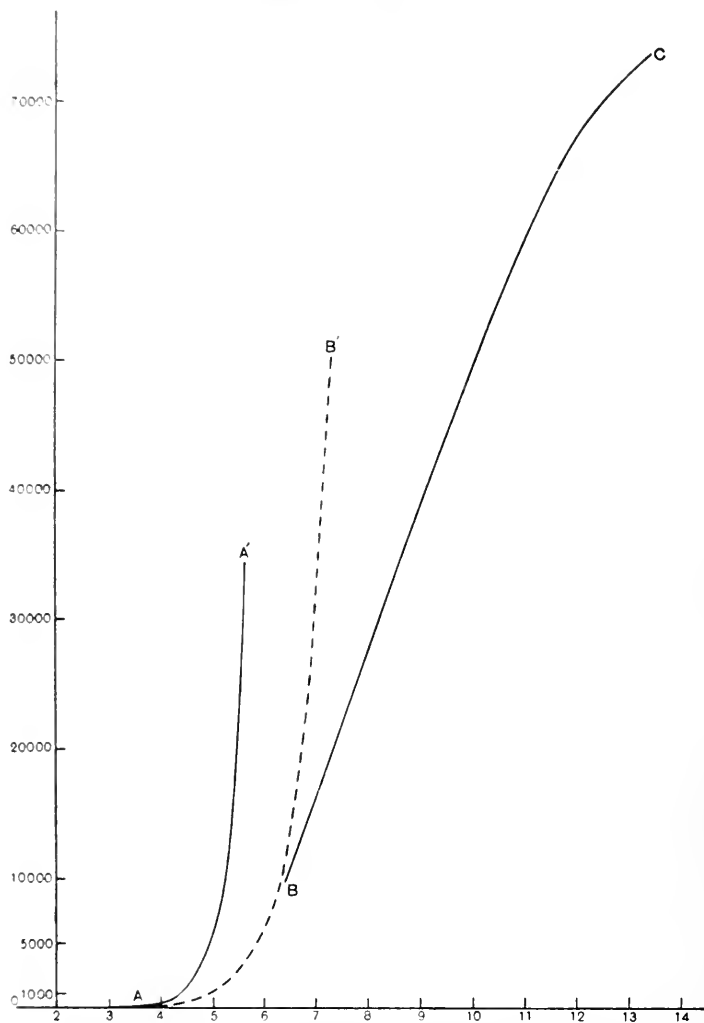


Chart IX. Ordinates. Bacilli. (Initial seeding taken as 1.)
Abcissae. Time in hours.

We see therefore that growth proceeds in those phases during each of which a definite law is maintained. Sufficient data are not at present

available to establish the law which holds during the 3rd phase, and consequently the point at which a maximum bacterial population would be reached.

The general form of the complete curve *OABC* suggests that possibly a frequency-curve might be got to fit all the data from the seeding onwards. This possibility, however, cannot be seriously entertained at present owing to the lack of data.

GENERAL CONCLUSIONS.

1. The phase of bacterial lag is a perfectly definite one, during which growth proceeds regularly from the period of seeding to the attainment of a minimum generation-time. During this phase time and bacilli are connected by an equation of the form $X^n = k \log \frac{Y}{s}$, where n and k are constants and s is the initial seeding.

2. At the close of the lag an entirely different law begins to hold and is maintained for another period of variable duration. During this phase the law $X = k \log Y$ holds and the generation-time remains constant throughout.

3. The logarithmic or 2nd phase is succeeded by the 3rd phase during which the generation-time gradually lengthens till it finally becomes infinite and no further growth occurs.

4. Growth is probably discontinuous in the sense that it conforms to different laws in the successive phases and it remains to be decided what theory of lag will most adequately accord with the numerical data and the mathematical laws derived therefrom. Various theories of the causation of lag have been discussed by one of us (Penfold, 1914) and special prominence has been given to two, viz. (1) based on variation of the bacterial cell and (2) based on a purely chemical analogy with ferment action generally and the importance of intermediate products in particular. Which of these two theories would best accord with the fact of discontinuous growth-laws cannot at present be decided in the absence of further experimental evidence of a crucial nature. In the meantime one cannot fail to be impressed by the analogy that exists between recently ascertained facts with regard to bacterial variation in a sugar-containing medium and the discontinuous growth-phases that occur in a medium of simple constitution. It may, for example, be assumed that during the lag-phase an organism is being selected out which can propagate itself with a constant minimum generation-time.

This selected strain holds the field during the second or logarithmic phase just as the selected dulcitate-fermenting *B. typhosus* variant holds the field after the initial period of selection is over. In the 3rd phase we again have competing strains, but here the mean result is of an inverse character and the mean generation-time progressively lengthens. (Phase of reversion.)

The hypothesis that variation processes are at work receives further support from the fact that a seeding taken during the lag-phase grows with diminished lag, while one taken during the 2nd phase proceeds to grow with practically no lag.

It is possible that more frequent observations at the boundary zones of these phases would shed further light on this problem.

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EXPERIMENTS ON THE AGGLUTINATION OF WATERY EXTRACTS OF *B. TYPHOSUS*.

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I. *Introduction.*

THE following observations on the agglutination of bacterial extracts were made in the hope of finding out whether the substances agglutinated by a specific serum and by acid were identical, and also with a view to throwing light on the nature of the process of agglutination by acids.

The conglomeration and subsequent sedimentation of the more or less invisible colloidal particles in a bacterial extract is often spoken of as a precipitation and distinguished from the agglutination of an emulsion of bacteria. Since, however, there appears to be no essential difference between the two processes whether they are brought about by specific serum or by acid, the term agglutination has been used in this paper to include the precipitation of clear bacterial extracts.

It was shown by Kraus (1897, B) that the phenomenon of agglutination by a specific serum still occurred when a filtered bacteria-free extract was used instead of an emulsion of the bacilli. He used filtrates of old broth cultures of *B. typhosus* and *V. cholerae*, and specific sera which were only slightly diluted.

Ch. Nicolle (1898) showed that a filtered watery extract of *B. typhosus*, *B. coli* or *V. cholerae* grown on agar was agglutinated by specific serum diluted 1/10. He also showed that in a watery extract to which specific serum had been added, suspended bacilli of quite a different kind from that used in preparing the extract were completely clumped. When for instance he mixed an extract of *B. coli* and an antiserum to this strain of *B. coli*, and to this mixture of antigen and serum he added an emulsion of *B. typhosus*, he obtained complete clumping of the latter. Further he showed that inorganic particles, *e.g.* finely divided talc in suspension were clumped in a mixture of bacillary extract (antigen) and its specific serum (agglutinin).

He did not use highly diluted sera nor dilutions of extracts, as the agglutination reaction was comparatively newly discovered at that time.

Paltauf (1897) and Kraus (1897 A) also put forward the view that the agglutination of bacteria was due to the formation of a kind of coagulum in the liquid, in which the bacteria were entangled and drawn together into clumps.

Kraus and von Pirquet (1902) produced further evidence of the identity of bacterial agglutination and the precipitation of bacterial extracts by serum. They used a specific serum, which in some cases was diluted as much as 1/150, in order to obtain the agglutination or precipitation of an extract, but a dilution of serum of 1/10 or 1/50 was more commonly used for this purpose.

Dean, H. R. (1912), showed that a watery extract diluted 1/160 would in some cases give a precipitate with specific serum 1/10. He also showed that on addition of a small quantity of the mid-piece (euglobulin) of guinea-pig's complement a precipitate was obtained with extract 1/640 and specific serum 1/10, whereas with this high dilution

of extract no precipitate was obtained on the addition of specific serum alone. He considered this enhanced result was due to the mid-piece increasing the size of the precipitate mechanically, and thus making it visible, and suggested that the action of the mid-piece in this case was similar to that of the conglutinin of ox-serum which was used by Bordet and Gay (1906) and Streng (1909) for enhancing or producing agglutination in mixtures of specific serum and red blood corpuscles, or specific serum and bacteria.

Michaelis (1911) described the agglutination of bacteria by acids. He used for this purpose regulator mixtures of acetic acid and acetate of sodium, lactic acid and lactate of sodium, etc., affording solutions of known hydrogen-ion concentration, and he showed that the kind of acid was unimportant so long as the correct hydrogen-ion concentration was present. He records the observation that at the optimum hydrogen-ion concentration for agglutination and even in slightly stronger acid mixtures no change in the electric charge of the bacteria could be observed by means of U-tube cataphoresis experiments. In commenting on the difference of behaviour in this respect of bacteria and suspensions of various proteins which have been examined, he suggested that the peculiarity of bacterial emulsions was due to the complex composition of bacteria and that the different constituents had isoelectric points corresponding to different concentrations of hydrogen-ions. Beniasch (1912) and Beintker (1912) extended Michaelis' agglutination experiments, corroborating his views, and maintained that the substances agglutinated by acid and by serum were identical, on the ground that the two reactions ran parallel in a series of strains tested. Michaelis and Davidsohn (1912) agglutinated a watery extract of *B. typhosus* with acid, removed the precipitate by centrifuging and redispersed it by neutralising. They found that the clear "solution" of the precipitate thus obtained was precipitable by serum like the original extract. They maintain that this proves the identity of the substances in a bacterial extract or emulsion which are agglutinable by serum and acid respectively. They assume that the optimum hydrogen-ion concentration for precipitation of the extract is also the isoelectric point, on the analogy of the proteins and other substances which they have previously examined, and they conclude that the substances which are agglutinable by serum and acid are identical and are precipitated by acids at their isoelectric point.

However, they appear only to have shown that the substance agglutinated by serum is carried down with the acid agglutinable substance,

when the requisite $[H^+]$ is present for the precipitation of the latter. Also they record no direct experiment to demonstrate the charge on the particles in an extract in solutions of varying $[H^+]$.

The work of Krumwiede and Pratt (1913) and of Sgalitzer (1913) must be mentioned, since their observations have a bearing on the agglutination by acid of mixtures of bacillary bodies and normal serum and of bacillary bodies and extract recorded in Section 7 of the following paper. Sgalitzer also records a large number of experiments on acid agglutination under different conditions. These workers describe methods of combined agglutination by acid and specific serum which they claim are more delicate than the use of acid or serum separately. Both sets of experiments were carried out by adding acid in the presence of the serum proteins. The effect therefore of the acid on the protein as well as on the bacilli makes the test a complicated one. Sgalitzer uses hydrochloric acid and therefore the $[H^+]$ cannot be accurately calculated. Krumwiede and Pratt use acid regulators and the effect of the salt (acetate or lactate) in the regulator on the sensitised bacilli must further complicate the action of the acid on the sensitised bacilli and on the serum proteins.

Michaelis and Davidsohn (1912) record experiments on the action of acid regulators on sensitised bacteria (after removal of the serum protein), which do not support the view that acid affects sensitised differently from unsensitised bacilli.

II. *Writer's experiments.*

(1) *Method of preparing bacterial extracts.*

The observations here recorded were all made with strains of *B. typhosus*. In order to be able to observe serum agglutination and acid agglutination with the same bacterial extract the bacilli from a 24 to 48 hrs. agar slope were washed off with 10 c.c. of distilled water and the resulting emulsion was centrifuged after standing for half an hour to an hour at the temperature of the laboratory. The supernatant liquid was again centrifuged once or twice till quite clear. In some cases a second active extract could be obtained by making up the deposit to its original volume with distilled water, allowing it to stand at room temperature for one or two days and again centrifuging. Chloroform was added to the emulsions and to the extract to prevent decomposition, this addition being found by preliminary experiment not to perceptibly influence the agglutination reactions.

It was observed that, independently of the age of the culture, different strains and different races of the same strain, and also the same strain on different occasions, yielded extracts of very different values.

Sometimes the bacteria in the first deposit obtained by centrifuging when re-emulsified no longer agglutinated with acid or serum and the extract obtained by centrifuging this emulsion after it had stood for a day or longer, gave no precipitate with either reagent. On other occasions the bacilli were still agglutinable by serum after washing two or three times in the original volume of fresh distilled water. On two or three occasions extracts were obtained bacteria-free by filtering through a porcelain filter, but it was usually considered sufficient to centrifuge the extract after it had stood for some hours, as by this means clear extracts could be obtained which showed only six or eight bacilli in a field of the microscope when a drop was examined with a Zeiss D objective.

The results with filtered extracts were quite similar to those with extracts obtained by centrifuging.

(2) *Agglutination of extracts by salts.*

It was found in the case of a strain of *B. typhosus*, an emulsion of which agglutinated without serum in sodium chloride, calcium chloride or lanthanum nitrate solution, that a watery extract was also agglutinated by these salts and it was found that the optimum concentration of salt for agglutination was approximately the same whether an emulsion of the bacilli or an extract was used. For this purpose only weak solutions of salts were used. In the cases of those strains which were examined the optimum concentration of sodium chloride was N/4 to N/8, of calcium chloride N/16 to N/32 and of lanthanum nitrate N/400 to N/800. See Table I.

(3) *Agglutination of extracts by acids.*

The regulator mixtures of known hydrogen-ion concentration recommended by Michaelis were used as a rule, but experiments were also made with dilute hydrochloric acid or sulphuric acid.

Regulator mixtures of acetate of sodium and acetic acid or of lactate of sodium and lactic acid were prepared in such a way that each acid solution contained 5 c.c. of normal acetate or lactate in 210 c.c. The solutions were numbered 1 to 10. The hydrogen-ion concentration or $[H^+]$ in each solution was twice that in the preceding solution of the series. See Table II.

TABLE I.

Agglutination of emulsion and extract of a "spontaneously" agglutinable strain of B. typhosus by salts.

NaCl	N 1	N 2	N 4	N 8	N 16	N/32	N/64	N/128	N/256	D. W.
Emulsion	-	-	+	-	-	-	-	-	-	-
Extract	-	+	+	-	-	-	-	-	-	-
CaCl ₂	N 1	N 2	N 4	N/8	N 16	N/32	N/64	N/128	N/256	D. W.
Emulsion	-	-	≠	≠	≡	≡	+	-	-	-
Extract	+	+	+	≠	≡	≡	+	-	-	-
La(N ₂ O ₅) ₃	N 200	N 400	N 800	N/1600	N/3200					D. W.
Emulsion	≡	≠	≡	≠	-					-
Extract	≡	+	+	-	-					-

TABLE II.

Agglutination by acid regulator mixtures of emulsions of three different strains of B. typhosus.

Regulator solution	10	9	8	7	6	5	4	
[H]	1.1×10^{-3}	5.6×10^{-4}	2.8×10^{-4}	1.4×10^{-4}	7.2×10^{-5}	3.6×10^{-5}	1.8×10^{-5}	
<i>B. typhosus</i> , Strain L	-	-	-	+	+	+	-	37° 15 mins.
	+	-	+	+	+	+	-	37° 30 mins.; room temp. 30 mins.
	+	+	+	+	+	+	-	37° 30 mins.; room temp. 20 hrs.
<i>B. typhosus</i> , Strain G	-	-	-	-	-	-	-	37° 15 mins.
	-	-	-	-	-	-	-	37° 30 mins.; room temp. 30 mins.
	-	-	-	-	-	-	-	37° 30 mins.; room temp. 20 hrs.
<i>B. typhosus</i> , Strain S	-	-	-	-	+	+	-	37° 15 mins.
	+	+	+	+	+	+	-	37° 30 mins.; room temp. 30 mins.
	+	+	+	+	+	+	-	37° 30 mins.; room temp. 20 hrs.

- = no agglutination, + = distinct agglutination, + and ++ = marked and complete agglutination, + s = slight agglutination.

If a series of tubes is put up containing the different solutions and an emulsion of *B. typhosus* in distilled water is added in equal quantity it is found that the optimum [H] for agglutination is almost invariably that corresponding to tube 5 or 6. The best time for making this observation varies but is usually after about 15 minutes at 37° C. and 15 minutes at the temperature of the laboratory in the case of

emulsions, but in the case of extracts two or three hours at room temperature after a short time in the incubator are often required to allow the formation of a visible precipitate. On standing for longer than the time required to show the optimum, *i.e.* for agglutination to appear in the first tube in which it occurs, further agglutination is observed in some of the tubes containing stronger acid, but seldom in any tube containing weaker acid than tube 5.

These effects were described by Michaelis and his co-workers, but they laid stress only on the optimum $[H^+]$ for agglutination.

As a rule agglutination is soon seen in tube 6 and about the same time it is also evident in tube 10 and sometimes to a less degree in tube 9. The agglutination in tubes 9 and 10 is usually of a different character, the clumps being in the form of fine granules, whereas in tubes 5 and 6 loose flocculi are seen. Eventually agglutination may occur in all the tubes from 5 to 10, but if they are observed from hour to hour a zone of absent agglutination is generally to be seen corresponding to tubes 8 or 9.

Table II shows the agglutination by acid of the emulsions of three strains of *B. typhosus*. Three observations at different intervals of time are recorded in each case. The later agglutination which occurs in higher $[H^+]$ than the optimum is of interest and shows the zone with diminished agglutination in tube 9.

The two optima and the zone of absent agglutination are perhaps seen best with bacilli which have been centrifuged down and re-emulsified (first deposit). When bacilli have been repeatedly washed with distilled water the emulsified deposit no longer agglutinates in acid solutions Nos. 5 and 6, but agglutination still occurs in solution No. 10. (Certain other bacteria including staphylococci and some strains of *B. coli* agglutinate first in tube 10, and later in tubes 9 and 8.) On the other hand a clear watery extract of *B. typhosus* as a rule agglutinates in solution No. 5, but not at all in No. 10. These observed facts appear to indicate that two substances, both agglutinable by acid, are present in whole emulsion of *B. typhosus*, one of which is soluble in water and has its optimum $[H^+]$ for agglutination in tube 5, and the other, which is closely adherent to the bodies of the bacilli, is agglutinated best in tube 10.

As a general statement it may be asserted that, although there is much variation in the behaviour of emulsions and extracts with acids, if a watery extract and well-washed bacilli from the same emulsion are compared, the substance whose optimum for agglutination is in No. 5

is most evident in the extract, while that whose optimum is in No. 10 is found more in the washed bacilli.

The original emulsion is sometimes not agglutinated in tubes 9, 10 and 11, although the usual agglutination occurs in tubes 5 and 6. This behaviour suggests the presence of a substance in the original emulsion which inhibits agglutination in tube 10. The substance may be merely a trace of agar or may be a real constituent of the bacilli. It is no doubt present in the first extract and may be in part that bacillary substance which is agglutinated in tube 5. It cannot be entirely composed of this agglutinable substance, since in the case of a strain of *B. typhosus*, e.g. strain "G," an emulsion of which is not agglutinated in any of the acid solutions 5 to 10, the bacilli after they have been washed are agglutinated in solution 10.

TABLE III.

Comparison of agglutination by acid of extracts and re-emulsified deposits.

Regulator acid solution—	10	9	8	7	6	5	4	D. W.
<i>B. typhosus</i> L, Extract 1	—	—	—	‡	‡	—	—	—
.. Extract 2	—	—	—	—	+	+	—	—
.. Deposit 1	‡‡	‡	‡	+	+	+	—	—
.. Deposit 4	‡	‡‡	‡	+	—	—	—	—
.. Extract 3	—	—	—	+	+	+	—	—
.. Deposit 3	—	‡	—	—	—	—	—	—

Table III shows the agglutination by acid of successive extracts and repeatedly washed bacilli of *B. typhosus* strain "L." Extract (1) and Deposit (1) were the clear supernatant fluid and the re-emulsified deposit obtained by centrifuging the original emulsion. Extracts (2) and (3) and Deposits (3) and (4) were obtained by re-centrifuging Deposit (1), removing the supernatant, re-emulsifying the fresh deposit, etc. The two different optima, for the extracts in tube 6, and for the washed bacilli in tube 9 or 10, are well shown.

Table IV also shows the different zones of agglutination by acid obtained with the original emulsion, the first and second extracts, and the first and second deposits (*i.e.* bacilli free from extract). Three observations on the agglutination are recorded after the tubes had stood for different lengths of time in the case of the emulsion and Deposit (1), and two observations on Extract (1). The last line in the table shows the reappearance of agglutination in tube 6 when Extract (2) is added to Deposit (2), and also shows the two optima with a zone of absent

agglutination in tubes 7, 8 and 9. Some observations on the electric charge carried by the bacilli and precipitate are also recorded and will be referred to later.

TABLE IV.

Agglutination by acid of the "whole emulsion," extracts and deposits from a strain of B. typhosus "S."

Acid solution—	12	11	10	9	8	7	6	5	4	D. W.	
<i>B. ty.</i> "S" Em.	—	—	—	—	s +	+	s +	—	—	—	} Agglutination.
	—	—	+	—	#	##	##	#	—	—	
	+	#	#	+	#	##	##	##	—	—	
	0	0	—	—	—	—	—	—	—	—	
											Electric charge.
„ Ex. 1	—	—	—	—	—	—	+ s	—	—	—	} Agglutination.
	—	—	—	—	+	##	##	—	—	—	
	—	—	—	—	—	—	—	—	—	—	
											Charge.
„ Dep. 1	+	—	—	—	—	—	—	—	—	—	} Agglutination.
	+	+	+	—	—	+ s	+	+ s	—	—	
	##	#	#	+	+ s	#	##	##	#	—	
	0	—	—	—	—	—	—	—	—	—	
											Charge.
„ Ex. 2	—	—	—	—	—	—	—	—	—	—	} Agglutination.
Dep. 2	+	#	##	+ s	—	—	—	—	—	—	
„ Ex. 2 }	—	+ s	+	—	—	—	#	—	—	—	
+ Dep. 2 }	—	+ s	+	—	—	—	#	—	—	—	

(4) *Agglutination of bacterial extract with specific serum, and its relation to agglutination by acids.*

In experiments with watery extracts and emulsions sufficient salt must be added to enable agglutination to take place after sensitisation by the agglutinins. It was found that 0.42 % of sodium chloride was about the optimum salt concentration for the agglutination of *B. typhosus* by specific serum. If ordinary salt solution (0.85 %) was used to dilute the serum and the watery emulsion or extract was added to dilutions of serum in equal parts sufficient salt was present. A smaller percentage of salt was however amply sufficient, and in some experiments only 0.2 % or 0.3 % of NaCl was used.

If a strain of *B. typhosus* is selected which agglutinates readily with a specific serum and an active clear watery extract is prepared, on the addition of dilute anti-typhoid serum a delicate fleecy precipitate is produced. If a powerful anti-typhoid serum (titre 1/50,000) is used, this agglutination or precipitation reaction can be obtained when the serum is diluted as much as three thousand or nine thousand times.

Kraus and most other workers appear to have obtained a precipitate only when comparatively strong serum, *e.g.* diluted to 1/50 or 1/150, was used. With a perfectly clear extract, the precipitate is very small when the serum is highly diluted, for the size of the precipitate depends in part on the amount of serum proteins precipitated. There is reason however to think that the specific substances may be present in sufficient quantity to produce a precipitate under favourable circumstances, even when the extract and serum are both diluted considerably beyond the point at which a visible precipitate ceases. (See Section 7.)

In Table V the results with the original emulsion, the extract and the deposit are compared. Two strains of *B. typhosus* were used. "L," a strain of medium agglutinability, and "G," which agglutinated less well with serum than "L" and never agglutinated at all with

TABLE V.

Agglutination of emulsion, extract and deposit of two strains of B. typhosus by serum and by acid.

	Antityphoid serum					Hydrochloric acid				
	1/1600	1/3200	1/6400	1/12800	Salt sol.	N/200	N/400	N/800	N/1600	D. W.
<i>B. ty.</i> "G" Em.	+++	+++	+	+	-	-	-	-	-	-
" Ex.	+++	+	-	-	-	-	-	-	-	-
" Dep.	+	+	-	-	-	+++	+	-	-	-
<i>B. ty.</i> "L" Em.	+++	+++	+++	+	-	+++	+	+	-	-
" Ex.	+	+	+	+	-	+++	+++	+	-	-
" Dep.	+	+	+	+	-	+++	+	+	-	-

acid regulators Nos. 5 to 7. The deposit of "G" showed agglutination in the absence of extract in the stronger acid solutions which probably corresponded to No. 10 or 11 regulator, but the [H⁺] of the tube marked HCl N/200 was not determined. The two optima for the case of "L" are not shown; probably because the gradations in successive dilutions of HCl are separated by too large intervals.

It has usually been found that if an emulsion agglutinates in a high dilution of serum, an extract obtained from this emulsion also agglutinates well, *i.e.* when the serum is highly diluted or the extract is diluted several times.

It is unusual to obtain a visible precipitate on the addition of acid or highly diluted serum to a clear extract which has been diluted more than four times. A precipitate is sometimes seen on addition of dilute specific serum (*e.g.* 1/3000) to a diluted extract although no precipitate is visible on addition of acid (No. 5). This may happen in the case of

an extract which undiluted readily agglutinates with acid. Specific serum therefore appears to be a more delicate test of the presence of extract than is acid.

TABLE VI.

Ex. <i>B. ty.</i>	<i>B. ty.</i> "L."				<i>B. ty.</i> "G"				Dilution of ext.
	1/2	1/4	1/8	1/16	1/2	1/4	1/8	1/16	
Acid mixture No. 6	+	-	-	-	-	-	-	-	
Anti-ty. serum	1/3200 +	+	+	+	+	+	+	-	

Table VI shows the greater sensitiveness of serum than acid as a test for the presence of agglutinable substance, but in this experiment the difference between the two reactions was unusually great in the case of strain "L." This was probably due to the use of rather strong serum and also to the fact that only one acid solution was used which may not have been the optimum. Strain "G" never agglutinated with acid No. 6.

A variant strain of *B. typhosus* was met with which did not agglutinate (either extract or emulsion) with serum or acid. The strain was however a descendant through agar cultures of a normal culture of *B. typhosus* and resembled the parent strain in its cultural and fermentative reactions. It is probable that in this case the failure to agglutinate was due to the absence of soluble bacillary substance from the liquid part of the emulsion. This suggests a probable explanation of the inagglutinability of some strains of *B. typhosus*. On the other hand some strains, *e.g.* strain "G," which do not agglutinate with acid (No. 5) are readily agglutinable by specific serum, though usually not quite so readily as other strains which are also agglutinated by acid. The writer has met with two such strains which were agglutinated by serum but not by acid.

Strains of *B. typhosus* also occur which are not agglutinated by specific serum, but which agglutinate in acid solution of the strength characteristic for this bacillus. (McIntosh and McQueen, 1914.) The record of such a strain completes the proof that the two properties of agglutinability by acid (Nos. 5 and 6) and by serum may vary independently. Probably this variability is concerned with at least two factors:

(1) The total amount of soluble bacterial substance in the liquid of the emulsion or extract; the bacilli of some strains appear to give up soluble material to water more readily than in the case of other strains.

(2) The relative amounts of the two substances agglutinable by acid or serum respectively (if these two substances are really distinct), or the relative degree of the property of agglutinability by acid or by serum possessed by the agglutinable substance (if there is only one agglutinable substance whose properties vary in different strains).

(5) *The effect of heating extracts and emulsions of B. typhosus.*

Porges and Prantschoff (1906) showed that if an emulsion of *B. typhosus* was heated to 80° C. it ceased to be agglutinable by serum and by certain electrolytes. After further heating to 100° C. however the agglutinability was restored. Porges (1905) also found that bacilli whose agglutinability had been destroyed by heating to 80° again became agglutinable if they were washed with salt solution, and he maintained that by this means some inhibiting substance was removed.

Beniasch (1912) found that if an emulsion of *B. typhosus* in distilled water was heated to 100° C. agglutination subsequently could be obtained with acid, but that the optimum $[H^+]$ was altered from 3.6×10^{-5} to 1.1×10^{-3} or 2.2×10^{-3} . He also found that *B. coli*, *B. dysenteriae* and certain other bacteria showed the same optimum after being boiled.

The present writer has repeated these experiments, and has also tested the effect on agglutination by acid of heating extract and washed bacilli to 80° and 100° with the following results :

Heating the whole emulsion for 20 or 30 minutes at 80° completely abolished the agglutination in acid solutions Nos. 5 and 6, *i.e.* $[H^+]$ 3.6×10^{-5} and 7.2×10^{-5} , whereas the agglutination in No. 10, *i.e.* $[H^+]$ 1.1×10^{-3} , was either absent or much diminished. After heating to 100° for 5 minutes good agglutination occurred in No. 10 but not in Nos. 5 or 6.

After being heated to 80° or 100° the extract was completely inagglutinable at either optimum. Heating the washed bacilli to 80° or 100° removed any agglutinability which the unheated re-emulsified deposit showed in tubes 5 and 6, but the effect on the agglutination in No. 10 was very slight.

Table VII shows the effect of heat on a "whole emulsion," an extract and a re-emulsified deposit of washed bacilli (Dep. 2). The bacilli, as in the other experiments, were from a culture of *B. typhosus*. Two observations at 5 hours and 20 hours are recorded in the case of the unheated "whole emulsion," as the two optima for agglutination are best shown in this way. Otherwise the observations were all made

TABLE VII.

The change in agglutinability by acid after heating emulsion, extract and re-emulsified deposit of B. typhosus.

Acid regulator mixtures—	11	10	9	8	7	6	5	4	D. W.	Time at temp. of room (preceded by 15 mins. at 37°)
Whole Emulsion (unheated)		—		—	+	+	++	—	—	5 hours
„ „ „		++	+	+	+	++	++	—	—	20 hours
„ „ 80° 30 mins.		+	—	—	—	—	—	—	—	„
„ „ 100° 5 mins.		++	+	—	—	—	—	—	—	„
Extract (unheated)	...	—	—	—	+	+	++	—	—	„
„ 80° 30 mins.	...	—	—	—	—	—	—	—	—	„
„ 100° 5 mins.	...	—	—	—	—	—	—	—	—	„
Dep. (2) (unheated)	...	+	+	+	—	—	+	—	—	„
„ 80° 20 mins.	...	+	+	+	—	—	—	—	—	„
„ 100° 5 mins.	...	+	+	+	+	—	—	—	—	„

at the end of 20 hours at the temperature of the room preceded by 15 minutes at 37° C. It appears that the effect of heating to 100° C. for four or five minutes is to destroy or render inactive the substance whose optimum [H⁺] for agglutination is in tube 5 or 6, whereas the effect on the agglutination in No. 10 of the substance peculiar to the washed bodies (Dep. 2) of the bacilli is very slight or nil. Heating to 80° for 20 or 30 minutes alters the substance whose optimum [H⁺] is in tube 5 or 6, so that it is no longer agglutinable by acid. It may be that this substance is so changed by heating to 80° that besides being rendered inagglutinable it also prevents agglutination of the bacilli in tube 10. In this case it would represent the inhibitory substance of Porges. The effect of this inhibitory substance is seen in Table VII in the case of the "whole emulsion" which has been heated to 80°; only slight agglutination confined to tube 10 occurred. The source of the inhibitory substance suggested above is made more probable by the very slight evidence of this inhibitory effect when the bacilli were washed before being heated to 80°. Table VII also shows that further heating to 100° renders the inhibitory substance inactive and good agglutination of the whole emulsion in tubes 9 and 10 again takes place.

It is also seen that heating the substance contained in the washed bacilli to 80° or 100° has little effect on their subsequent agglutinability by acid, which remains as before.

(6) *The part played by the agglutinable substance in the extract in the agglutination of ordinary bacillary emulsions.*

(a) *Interbacillary substance in the clumps.* If an extract which has been centrifuged till very few bacilli remain in suspension (e.g. four or five in the field of an oil-immersion objective, in a dried drop on a slide) is agglutinated with acid or serum and the process watched in a microscope cell with dark ground illumination, a very delicate, loose, fleecy cloud is seen to appear in which are entangled any bacilli or particles of dust present. The whole "agglutinum" appears to contract slightly after it has been formed. It is stained slightly if suspended in a weak watery solution of fuchsin and then appears as a delicate network or tangle with highly refractile and more deeply stained points corresponding to the nodes in the network. If a drop is allowed to dry on a slide and stained as a dry film the characteristic appearance is lost, but a very finely granular background is stained by fuchsin. If Leishman's stain is used the granules are stained a faint pink and a very few blue bacilli are seen amongst the granules. Only three or four bacilli each about two or three microns long may be seen in a mass of granules corresponding to an "agglutinum" about 20×15 microns. The frame-work of the "agglutinum" corresponds to the interbacillary substance described by Löwit (1913) as occurring in a clump of bacilli agglutinated with specific serum.

(b) *Agglutination of indifferent particles with extract, and conglutination.* If a clear watery extract of *B. typhosus* is mixed with an emulsion of well-washed *B. typhosus* (rendered inagglutinable by washing) and acid solution No. 5 or dilute anti-typhoid serum is added, the bacilli are agglutinated in the same way as if the original emulsion had been used. If the bacilli in the mixture are too numerous then agglutination is incomplete. The same effect is produced if a thin emulsion of *B. coli* or *B. acidi lactici*, etc. is added to an extract of *B. typhosus* instead of washed *B. typhosus*.

It was also found that a suspension of staphylococci, kieselguhr or animal charcoal was agglutinated in the same way, but washed *B. typhosus* or *B. coli* were agglutinated better than staphylococci or inorganic particles.

If an active extract is used the anti-serum required to produce agglutination may be highly diluted (see Table VIII). If the extract is diluted with distilled water till no precipitate is visible on addition of acid or weak specific serum, agglutination and a visible precipitate

can be obtained with the same strength of extract, etc. if in addition washed bacilli are present, although these latter alone are quite inagglutinable with serum or in No. 5 solution.

In Table VIII is shown the agglutination of a *B. coli* emulsion, (1) by a mixture of diluted *B. typhosus* extract and anti-typhosus serum in three different dilutions, and (2) by diluted *B. typhosus* extract and acid solution No. 5. Equal parts of the three reagents extract, *B. coli* emulsion or distilled water and in addition a similar quantity of serum or acid were placed in each tube. The extract was quite clear and did not contain constituents for a precipitate in sufficient quantity to enable the reaction to become visible unless *B. coli* was

TABLE VIII.

The agglutination of varying dilutions of B. typhosus extract by serum and acid. The use of B. coli as an indicator.

Dilutions of <i>B. ty.</i> Extract—	1/3	1/6	1/12	1/24	1/48	1/96	D. W.
Anti-ty. serum 1/3000							
<i>B. coli</i> Emulsion	##	##	##	#	+	+	—
Distilled Water	—	—	—	—	—	—	—
Anti-ty. serum 1/9000							
<i>B. coli</i> Emulsion	#	#	#	+	+	+	—
Distilled Water	—	—	—	—	—	—	—
Anti-ty. serum 1/27,000							
<i>B. coli</i> Emulsion	#	#	+	+	+	+	—
Distilled Water	—	—	—	—	—	—	—
Acid Solution No. 5							
<i>B. coli</i> Emulsion	#	+	+	—	—	—	—
Distilled Water	—	—	—	—	—	—	—

0.5 c.c. of extract dilution, and anti-serum dilution or acid in each tube, and in addition either *B. coli* emulsion 0.5 c.c. or D. W. 0.5 c.c.

added as an indicator. The extract nevertheless was so potent that even when diluted 1/96 macroscopic agglutination of *B. coli* was obtained on addition of serum 1/27,000. The absence of agglutination of the *B. coli* by the serum or acid without extract is shown in the last column in each case.

Table IX shows similar results to those in Table VIII. In these experiments (1) the three alternatives distilled water, emulsion of kieselguhr or emulsion of *B. coli* were added to mixtures of (2) *B. typhosus* extract in varying dilutions, and (3) anti-typhosus serum 1/1000 or acid solution No. 5.

(1), (2) and (3) were added to the tubes in equal quantities, *i.e.* 0.5 c.c. of each.

TABLE IX.

Agglutination of B. coli and kieselguhr by B. typhosus extract with serum or acid.

Dilutions of <i>B. ty.</i> Extract—1/2	1/4	1/8	1/16	1/32	1/64	1/128	D. W.	1/2
Anti-ty. serum 1/1000	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.0
Distilled Water	+	+	—	—	—	—	—	—
Kieselguhr	+++	+	+	+	—	—	—	—
<i>B. coli</i> Emulsion	+++	+	+	+	—	—	—	—
Acid Solution No. 5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.0
Distilled Water	+	+	—	—	—	—	—	—
Kieselguhr	+	+	—	—	—	—	—	—
<i>B. coli</i> Emulsion	+	+++	+++	+++	—	—	—	—

These experiments show that the extract and serum may be present in sufficient quantities to cause agglutination, but the reaction cannot be demonstrated without the presence of some material which will make the precipitate sufficiently large to be seen. An extract can sometimes be diluted four or eight or more times beyond the minimum strength in which the agglutination is still visible when the extract is used alone, and yet a good agglutination of indifferent particles can be obtained on the addition of serum or acid.

In this way *B. coli* or kieselguhr can be used as an indicator to show the presence of an agglutination reaction, when the extract is too weak to form a visible precipitate. For this purpose a thin suspension of the indifferent particles is most suitable.

The strain of *B. coli* which was generally used for this purpose agglutinated in acid No. 10 and sometimes also in 9 and 8 after long standing, but never in 5 or 6.

After addition of *B. typhosus* extract to the *B. coli* emulsion agglutination was sometimes confined to tube 5 and no agglutination occurred in tubes 9 and 10. In this case the *B. typhosus* extract appeared to act as a "protective colloid" and each whole particle (*B. coli* + *B. typhosus* extract) behaved as though it were composed of the extract. An adsorbed coating of each bacillus with *B. typhosus* colloid being assumed as probable (see Table XI, also Table II *B. typhosus* "L").

If normal serum (horse or rabbit) in a dilution of 1/50 to 1/60 was added to extract the precipitate on addition of specific serum 1/9000 was increased, in this case the globulin of the normal serum probably acted by increasing the bulk of the precipitate, as occurs in the ordinary precipitin reaction when strong serum is used. The addition therefore of strong normal serum can make visible an agglutination reaction

which cannot be seen when only dilute typhoid extract and dilute anti-typhoid serum are used. This increased precipitate on addition of normal serum is probably of the same kind as that described under the name of conglutination by Bordet and Streng as occurring on the addition of ox serum.

A similar increase in the precipitate was found by Dean to occur when mid-piece of guinea-pig complement was added to dilute typhoid extract and dilute anti-typhoid serum.

A low dilution of agglutinating serum may therefore be quite unnecessary in order to produce a precipitate with a bacterial extract. High dilutions of the specific serum may be used if enough indifferent substance (*e.g.* normal serum, indifferent bacteria, etc.) is present to make a sufficiently bulky precipitate.

(7) *The relation of electric charge to the agglutination of bacterial extracts.*

If acid, slightly stronger than the optimum for agglutination, is added to an emulsion of *B. typhosus*, *e.g.* regulator mixture 8 or 9, the bacilli remain dispersed for a variable length of time, often for many hours, or they may not become agglutinated at all. The bacilli dispersed in neutral or alkaline solutions always have a negative charge, that is they can be shown to move to the positive electrodes in a cataphoresis experiment.

It was expected by Michaelis and his co-workers that in a solution of optimum acidity for agglutination the charge would be nil (isoelectric point) and that in higher concentrations of acid when the bacilli are again dispersed that the charge on the bacilli would be positive, on the analogy of particles of serum protein, etc. These workers have however failed to demonstrate any change of charge on the bacilli. They showed by U-tube cataphoresis experiments that the bacilli always moved to the positive pole in solutions 5, 6, 7, the optimum acidity for agglutination, and also in stronger acid Nos. 8 and 9 as they do in weaker acids and in alkaline liquids. The writer has repeatedly confirmed these observations, using a microscope cell with platinum electrodes such as was used by Chick and Martin (1912) in their experiments on serum proteins.

Michaelis explains these results by pointing out that the bacilli are made up of numerous heterogeneous particles and suggests that they do not all become isoelectric at the same point of hydrogen-ion concentration.

It seemed possible to the writer that, since the agglutination in No. 5 was due to some substance contained in a watery extract, and

agglutination in No. 10 to a substance which remained in the bacillary bodies after repeated washing, then by use of an extract it might be possible to obtain the substance agglutinable in No. 5 in a state of comparative purity, and in this way the agglutination optimum and the isoelectric point might be shown to be identical. On making the experiment it was found that even when a perfectly clear extract was used the agglutinated particles in tubes 5, 6, 7, 8, and 9 were all negatively charged, as in tubes 1 to 4. In tubes 8 and 9, in which agglutination did not appear, the very few particles seen moving under the microscope may not have been composed of the substance under investigation, as this may have been completely dispersed in this degree of acidity, but the negative charge observed in tubes 5, 6, and 7 was determined by watching the movement of clumped particles, which were undoubtedly composed (at least in part) of the substance which is characteristic of *B. typhosus* and is agglutinated in tubes 5 and 6.

It was found however that in emulsions and in re-emulsified deposits the substance agglutinated in tube 10 became isoelectric (no movement to either pole) about this hydrogen-ion concentration, and sometimes a positive charge could be demonstrated on bacilli in tube 10 or 11. Also in HCl N/100 the bacilli can be shown to have a positive charge, and in slightly weaker acid to be isoelectric, moving toward neither electrode.

In Table IV are recorded observations as to the charge on the bacilli in an original emulsion and in a re-emulsified deposit (Dep. 1) and also on the particles seen in a first extract (Ext. 1). It is shown that the particles in the extract remain negatively charged throughout, whilst the bacilli in the "whole emulsion" and in the deposit become isoelectric in tube 11 or 12, *i.e.* in $[H^+]$ about 2.2×10^{-3} .

Table X shows the agglutination with acid of emulsions of washed bacilli of a strain of *B. typhosus*, and the charge carried by the bacilli in the different tubes. Both regulator mixtures and hydrochloric acid were used. The HCl was diluted so that each tube contained two-thirds of the acid in the preceding tube. It is seen that the bacilli became isoelectric at or about the optimum for agglutination and a positive charge was demonstrated in the tube containing HCl N/100. The agglutination only occurred in the zone corresponding to tubes 9 and 10 and not at all in the zone with lower $[H^+]$, as shown by the series of tubes with regulator mixtures.

The experiments recorded in Tables IV and X show that the optimum $[H^+]$ for agglutination and the isoelectric points approximately coincide for the washed bacilli, but no change of charge is associated with the

agglutination of the substance which is agglutinated in Nos. 5 and 6.

TABLE X.

Agglutination with acid regulator mixtures and with dilute HCl of the re-emulsified washed bacilli of a strain of B. typhosus. The electric charge on the bacilli is also recorded.

Lactic acid regulator	12	11	10	9	7	6	5	4	D. W.	
<i>B. ty.</i> , Dep. 2	+	+	###	###	+s	-	-	-	-	Agglutination.
" "	0	0	-	-	-	-	-	-	-	Charge.
HCl	N/100	N/150	N/225	N/337	N/505	N/757	N/1135	N/1702	N/2553	D. W.
<i>B. ty.</i> , Dep. 2	+	###	###	+s	+s	-	-	-	-	Agglutination.
" "	+	0	0	-	-	-	-	-	-	Charge.

D. W.=distilled water; agglutination ###, #, +, +s and - =complete, marked, distinct, slight and absent agglutination; charge +, 0, - =positive, isoelectric and negative charge.

As stated above, if a clear watery extract of *B. typhosus* is added to a weak watery solution of *B. coli* and the mixture put into tubes containing different regulator mixtures, agglutination occurs in tubes 5 and 6, or in 5, 6, 7, and 8, whereas when *B. coli* emulsion is present alone, the agglutination is in tube 10 or 8, 9, and 10. A very similar effect as regards agglutination may be produced by adding weak serum (*e.g.* normal horse serum) instead of *B. typhosus* extract. Agglutination then occurs in tubes 4 and 5, or in tubes 4, 5, 6, 7, and 8. If serum 1/300 is used the zone of agglutination is narrowed to tubes 4 and 5, if serum 1/1500, more tubes are involved. Thus the appearance of the tubes is nearly the same whether *B. typhosus* extract or serum 1/1500 is added. When however the electric charge on the bacilli is examined a very marked contrast is observed. In the series containing serum the bacilli move to the anode (negative charge) in distilled water and in tube 3 or weaker acid, but move to the kathode (positive charge) in tubes 8, 9, and 10. In tubes 4 and 5 no movement occurs, and in tubes 6 and 7 the bacilli are also isoelectric or they may move to the kathode according to the strength of serum employed.

Table XI shows an experiment of this kind; the influence of *B. typhosus* extract and of diluted normal serum on the agglutination and on the charge carried by *B. coli* is well shown. The agglutination optimum in the presence of serum corresponds to the isoelectric point, the dispersal in tube 3 and in distilled water corresponds to a negative charge and the dispersal in tubes 8 to 10 to a positive charge. On the other hand, though a similar appearance of the tubes, as regards agglutination and dispersal, is seen in the series of tubes which contain extract

TABLE XI.

Agglutination of washed *B. coli* by acid (1) with distilled water, (2) with extract of *B. typhosus*, (3) with normal horse serum 1:1500. The charge on the bacilli is also recorded.

Regulator acid mixture	10	9	8	7	6	5	4	3	D. W.	
(1) <i>B. coli</i> - D. W.		+	+	+	-	-	-	-	-	Agglutination.
.. ..		0	-	-	-	-	-	-	-	Charge.
(2) <i>B. coli</i> - <i>B. ty.</i> Ext.	-	-	+	++	++	+	-	-	-	Agglutination.
.. ..	0	-	-	-	-	-	-	-	-	Charge.
(3) <i>B. coli</i> - Serum 1:1500	-	-	+	++	++	++	+	-	-	Agglutination.
.. ..	+	+	+	0	0	0	0	-	-	Charge.

Agglutination ++, +, +, - = complete, marked, distinct and no agglutination.
Charge +, 0, - = positive, no movement, and negative charge.

instead of serum, the charge on the bacilli in these tubes is negative in tubes 3 to 9, *i.e.* throughout the region of agglutination. If Michaelis' hypothesis with regard to the negative charge through such a series of tubes is correct and the substance in the extract which agglutinates in tube 5 is isoelectric in this [H], then the charge carried by this substance must be so weak as not to influence the movement of the particles which are seen to move to the anode in tubes 5 to 10. And since the same phenomenon of movement of all visible particles to the anode is seen throughout the series of tubes when extract of *B. typhosus* is used alone, this extract, on this hypothesis, must contain two substances, (1) agglutinable in 5 with its isoelectric point in 5 or weaker acid, (2) with isoelectric point in 9 or stronger acid.

III. SUMMARY.

The experiments detailed above have already been discussed in the sections in which they have been recorded. The following is a brief summary of the results obtained.

(1) In many ways a clear watery extract of *B. typhosus* behaves like an ordinary emulsion of the bacilli. This is true in a general sense in respect of agglutination by serum, and the property of agglutinability by weak salts, which some emulsions of *B. typhosus* possess, is also exhibited by a watery extract of the same strain.

(2) The optimum hydrogen-ion concentration for the agglutination of an emulsion is also approximately the optimum for the agglutination of a watery extract made from the same emulsion. The washed bacilli and the original emulsion show a second optimum. Agglutination appears later at this secondary optimum which is in stronger acid. There is a zone of absent or deficient agglutination in solutions of

intermediate strength. The lower $[H^+]$ of the two is the characteristic optimum for *B. typhosus*, i.e. about 3.6×10^{-5} . The second optimum $[H^+]$, i.e. 1.1×10^{-3} to 2.2×10^{-3} , is approximately the same as the optimum for the agglutination of emulsions of many different bacteria, e.g. some strains of *B. coli*, staphylococcus, etc., and moreover is about the optimum for the agglutination of an emulsion of *B. typhosus* after it has been heated to 100°C .

These two optima appear to indicate the presence of two acid agglutinable substances in an emulsion of *B. typhosus*, (a) which is characteristic of this bacillus and is present in a watery extract (this substance is rendered inagglutinable by heating to 80°C . for 30 minutes or to 100°C . for five minutes, or longer, e.g. one hour), and (b) which is present in the bodies of the bacilli after repeated washing and which is not characteristic of *B. typhosus*, but has characters shared by similar substances in other bacilli; it resists heating to 100°C . for five minutes and is apparently little, if at all, affected by heating to 80°C . for half an hour.

(3) The substance agglutinable by serum and that agglutinable by acid No. 5, i.e. $[H^+] 3.6 \times 10^{-5}$, are both present in a watery extract and can both be completely removed from the bacillary bodies by washing with distilled water. The serum-agglutinable substance is however less easily completely removed. This apparent difference is perhaps due to the fact that serum is a more delicate reagent for testing for the presence of agglutinable substance. If an extract is diluted it will give a precipitate in higher dilution with serum than with acid and the bacillary bodies when repeatedly washed often react with serum after the acid agglutination in solution 5 or 6 has ceased to appear. These substances agglutinable by acid and by serum are however closely associated and are both precipitated by acid No. 5 or 6.

The identity of the two substances has however not been proved and the following considerations are very strongly opposed to this assumption, (a) a considerable number of strains of *B. typhosus* are agglutinable by serum and not by acid; (b) strains have been described which are agglutinable by acid No. 5 or 6 but not by serum; (c) the diminished agglutinability by serum exhibited by some strains which are inagglutinable by acid is apparently due to a total diminution of the "soluble" or extractible substance; (d) the substance agglutinable by acid, tube 5 or 6, i.e. $[H^+] 3.6 \times 10^{-5}$, is rendered inagglutinable by heating to 100°C . for five minutes, whereas Porges and Prantschoff (1906) have shown that the serum agglutinable substance is still active after being boiled.

It is not possible at present to say for certain whether the substances agglutinable by serum and acid are identical or distinct. If they are identical and there is only a single substance, its agglutination properties vary independently in different strains of *B. typhosus*.

(4) The importance for agglutination by serum of the substances in "solution" in the liquid part of a bacterial emulsion, and the impossibility of distinguishing between agglutination and the precipitation of bacterial extracts by serum, were first pointed out by Kraus and Paltauf, and by Kraus and v. Pirquet.

Their view is confirmed and extended to the agglutination by acids Nos. 5 and 6 by the result of some of the experiments described above. The facts adduced in support of the view that agglutination is essentially the formation of a coagulum in the liquid part of an emulsion are the following :

(a) The property of agglutination may be completely removed from *B. typhosus* by washing with water ; the resulting washed bacterial bodies no longer agglutinate with specific serum or in acid solution Nos. 5 to 7.

(b) The washings of the bacteria or watery extracts are agglutinable by specific serum or by acid solution No. 5 or 6.

(c) The watery extract even in very small quantities when added to the washed bacilli, indifferent bacilli or other finely divided particles, confers on them the property of being agglutinated by specific serum or acid solution 5 or 6.

(d) The extract reacts with highly diluted serum, *e.g.* 1/27,000, and may be considerably diluted, *e.g.* 1/96, without entirely losing its agglutinable properties with serum or acid.

(e) In order to demonstrate the presence of agglutinable substance in very low concentration (especially if highly diluted serum or if acid is used) it is necessary to add some indifferent substance, *e.g.* *B. coli*, kieselguhr, globulin, etc. in order to increase the bulk of the precipitate.

(f) The extract or successive extracts appear to contain all the characteristic agglutinable substance of the original emulsion. This is shown by the delicacy of the reaction, and the small amount of extract required to agglutinate an indicator, *e.g.* *B. coli*. The demonstration of the reaction with highly diluted serum 1/27,000 and much diluted extract 1/96 appears to be an advance on the work of previous writers. Nicolle did not use indifferent particles with high dilutions of serum or of extract, and the only other use of an indicator under such conditions appears to be the use of conglutinin by Bordet and Gay, and Streng, and of mid-piece by Dean.

(g) The microscopic demonstration of the coagulum formed in the process of agglutination has been made easy by taking a watery extract containing very few bacilli, *i.e.* ordinary emulsion from which most of the bacilli have been removed by centrifuging. After adding dilute specific serum or acid the formation of the coagulum may be watched in a microscope cell with dark ground illumination or by adding dilute fuchsin or eosin to the microscope cell.

IV. CONCLUSIONS.

(1) Emulsions of *B. typhosus* in distilled water contain two substances agglutinable by acid which have two different optima of hydrogen-ion concentration for agglutination.

(2) One of the substances, (*a*), which is contained in a clear watery extract has an optimum $[H^+]$ of 3.6×10^{-5} to 7.2×10^{-5} . The other, (*b*), is contained in the thoroughly washed bacterial bodies; its optimum $[H^+]$ is about 1.1×10^{-3} .

(3) Substance (*a*) is rendered inagglutinable by being heated to 80°C. for 30 minutes or 100°C. for five minutes; substance (*b*) is unaffected by heating to 80°C. or 100°C. for similar periods.

(4) The substance in an emulsion of *B. typhosus* which is agglutinable by specific serum is closely associated with the substance whose optimum $[H^+]$ for agglutination is 3.6×10^{-5} . The identity of these two substances is however on the whole improbable.

(5) The agglutination of an ordinary emulsion of *B. typhosus* by specific serum or acid of $[H^+] 3.6 \times 10^{-5}$ is brought about (practically entirely) by the substance which passes out of the bodies of the bacilli into the surrounding liquid, *i.e.* the view of Kraus, v. Paltauf and Nicolle as regards serum agglutination is confirmed by experiments with dilute extract and highly dilute serum, and its application is extended to agglutination by acid.

(6) Acid of $[H^+] 3.6 \times 10^{-5}$ or high dilutions of specific serum, *e.g.* 1/27,000, will cause an agglutination reaction in much diluted clear watery extract (*e.g.* 1/96) of *B. typhosus*, but the addition of indifferent particles is necessary to produce a visible agglutination or precipitate.

(7) There is no direct evidence of change of electric charge associated with the agglutination by acid of the substance in an emulsion or extract whose optimum for agglutination is 3.6×10^{-5} .

The observations of Michaelis and Beniasch on the cataphoresis of emulsions have been confirmed microscopically and found to apply to extracts also.

(8) Washed bacillary bodies of *B. typhosus* become isoelectric in $[H^+] 1.1 \times 10^{-3}$ or 2.2×10^{-3} , i.e. they do not move towards either electrode in this $[H^+]$, and move to the kathode in stronger $[H^+]$.

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REPORT TO THE LOCAL GOVERNMENT BOARD ON AN ENQUIRY INTO RAT PLAGUE IN EAST ANGLIA DURING THE PERIOD JULY— OCTOBER, 1911¹.

BY DRS A. EASTWOOD AND F. GRIFFITH.

(With Map.)

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INTRODUCTION.

THIS enquiry was conducted in accordance with the instructions of the Board, who directed that attention should first be confined to the area where plague-infected rats had previously been found and that, if such rats were again discovered, an endeavour should then be made to determine the limits of the infection. The area shown by previous investigations to have been infected comprised the rural districts of Samford and Woodbridge, the urban districts of Woodbridge and of Felixstowe and Walton, and the borough of Ipswich. The present enquiry commenced on July 1st, and was continued until October 31st, 1911.

ORGANISATION.

Police Assistance in Rat Collection.

The Board obtained, through the Home Office, the assistance of Captain Mayne, Chief Constable of East Suffolk, throughout the enquiry. Captain Mayne issued instructions to the following effect :

(1) The police officers throughout East Suffolk were to make confidential enquiries and then to report answers to the following questions :
 (a) Are rats known to be dying in any part of the officer's district ?
 (b) If so, in what parishes and on whose premises ? (c) What is the supposed cause of death ? (d) Has any sort of rat poison or virus been recently laid down ? (e) Any other information ?

(2) The officers in charge of every police station in that part of East Suffolk selected by the Board for the collection of rats were requested (a) to ascertain, by local enquiry, in what parts of their districts rats were likely to be found and to inform the Board's rat-catchers accordingly, (b) to receive at the police station all rats brought in by these rat-catchers, (c) to urge all residents in their districts to

catch rats and bring them in to the police station, and to pay, on behalf of the Board, 2d. per rat, (d) to disinfect all rats brought in, (e) to see that a disc bearing a number was attached to each rat, (f) to fill in for each rat a card of identification, bearing specified particulars, and (g) to forward the rats and the cards to the Municipal Laboratory at Ipswich.

When it was found necessary to extend the area of enquiry into portions of West Suffolk and the north of Essex, similar assistance was obtained, through the Home Office, from the Chief Constables of West Suffolk, Essex, and Colchester.

Arrangements by Mr Huddart.

Mr Huddart, Assistant Inspector to the Board, (1) made all arrangements with the Chief Constables and their superintendents for the work of the police, (2) engaged rat-catchers and assigned them work week by week, (3) obtained and distributed all material (disinfectants, cards, boxes, rat-traps, etc.) required by the police and the rat-catchers, (4) visited all the police stations and explained to the constables what was required, (5) made periodical tours of inspection throughout the enquiry for the purposes of supervising the rat-catchers and keeping in touch with the work of the police, and (6) took charge of the clerical work in connection with the enquiry.

The Ipswich Laboratory.

On the recommendation of Dr Pringle, Medical Officer of Health for Ipswich, the Council of that Borough placed their Municipal Laboratory gratuitously at the disposal of the Board throughout the enquiry. All rats were received and dissected in this laboratory by Drs G. H. Macalister and R. St John Brooks, who were engaged by the Board from July 1st to October 31st for the performance of this work. The routine clerical work was conducted in a room adjacent to but separate from the laboratory by Mr Leach, a temporary clerk, who worked under the direction of Mr Huddart.

The Board's Pathological Laboratory.

In the case of any rats which on post-mortem examination did not appear to Drs Macalister and Brooks to be free from suspicion of plague, the liver, spleen, and any other suspected material from each of these animals were sent to the Board's laboratory for diagnosis. They were

there examined microscopically, culturally, and by animal inoculation. Dr Klein acted as consultant in cases where it was thought that the diagnosis needed his confirmation.

Routine.

When a rat was caught by one of the Board's rat-catchers he attached to it with string a perforated circular metal disc bearing a stamped number. This number and the place where the rat was caught he recorded in a note-book. It was to his interest to keep these records accurately, as his weekly claims for payment were based on them. He caught his rats with the aid of ferrets, dogs, and traps, but was not allowed to use poison. In addition to the rats so obtained he added to his collection any dead rats he could find; these latter were labelled and recorded in the same way as the caught rats, and, in addition, a piece of coloured tape was tied to each as a distinctive mark.

He brought in his day's bag, in which he was not permitted to include any rats less than half-grown, to the nearest police station. There the constable disinfected them in 2 per cent. lysol and entered on a separate card for each rat the following particulars: (1) the number on the metal disc (termed "local number"); (2) the date; (3) name of catcher; (4) parish where caught or found; (5) exact place where caught or found; (6) whether caught or found dead; (7) whether the occupier had previously laid down virus; (8) if so, when and what kind.

When rats were brought in by voluntary workers, the constable affixed to each a disc bearing a "local number," tied coloured tape to any found dead, and then dealt with them in the same way as with those brought in by the professionals.

The constable packed the rats in tin boxes, each of which was enclosed within an outer wooden box, and forwarded them, generally by carrier, to the Ipswich laboratory. The cards bearing details of each rat were sent to the laboratory by post.

At Ipswich the cards were received in the clerk's room, the rat-boxes in the laboratory.

When the examination of each rat was completed, the circular disc bearing the local number was removed by cutting the string attaching it to the rat, and a square disc bearing a serial number was attached by wire to the circular disc. The serial numbers ran in sequence from 1 upwards, and registered the total number of rats received in the laboratory. The pairs of circular and square discs were collected in two lots,

one bearing the numbers of the rats found free from plague, the other bearing the numbers of those reserved for further examination. After being sterilised, these batches of discs were sent up to the clerk's room. No articles of any sort which might have been in contact with infective material were sent up to the clerk's room without being sterilised.

On receiving the pairs of discs, the clerk identified each card by its local number and copied on to it the corresponding serial number. Each rat was then entered up, according to its serial number, as "normal" (*i.e.* free from plague) or "reserved" (*i.e.* specimens sent to the Board's laboratory for examination). At the end of each day's work the clerk drew up a detailed statement of all the rats examined during the day, giving the names of the parishes from which the rats were received, the numbers of rats received from each, the serial numbers of all "normal" and all "reserved" rats and of all rats found dead, and, in the case of each "reserved" rat and of every rat found dead, the exact address where it was obtained. One copy of this information was posted each evening to the Board's laboratory and another to Mr Huddart.

In the case of each "reserved" rat Drs Macalister and Brooks sent to the Board's laboratory the liver, spleen and any other material considered suspicious, together with a full account of the post-mortem appearances of the rat, including microscopic evidence obtained from smear preparations. This material and information, and also the clerk's daily record, were received the next morning at the Board's laboratory. The specimens were there investigated microscopically, culturally, and by animal inoculation. When a case of plague was fully established, the Board were at once notified of the fact and of the exact place whence the rat was obtained. At the same time Mr Huddart was asked to stop further supplies from the parish whence the rat was obtained, to notify the occupier of the premises where the rat was found of the presence of rat-plague, to warn him of the danger, and to advise him to take energetic measures to destroy any rats on his premises.

BACTERIOLOGICAL DIAGNOSIS IN THE BOARD'S LABORATORY.

Methods.

The routine method of staining film preparations from tissues, fluids, or cultures was as follows: After drying in the air and then fixing for two minutes in absolute alcohol, the smears were stained, as

recommended by Dr Klein, with Czinzinski's mixture of methylene blue and eosin. The formula for this mixture is:

Methylene blue (conc. aqueous solution)	..	50 c.c.
Eosin (soluble in alcohol)5 gm.
Alcohol (absolute)	70 c.c.
Water (distilled)	130 c.c.

The stain was applied for at least five minutes, and at the beginning and the end of this period was warmed over the flame until steam rose. The specimens were then washed in water, dried, and mounted in Canada balsam.

For the isolation of cultures slanted agar tubes and agar plates were employed, the medium consisting of ordinary nutrient agar made with beef broth, reaction + 10 to phenolphthalein. The surface of the agar was fairly dry. In the earlier part of the investigation some use was made of MacConkey's neutral-red, bile salt medium, with the addition of mannite or lactose. It was found, however, by comparative experiments with plague-infected tissue, that growth on a bile salt medium was less certain and slower than on agar, and that the bile salt had some inhibitory effect, fewer plague colonies being produced than on ordinary nutrient agar plates inoculated with the same dose of the same material. Again, the fact that intestinal bacteria, the growth of which was not inhibited by bile salt, were commonly present in such contaminated and decomposed tissues as had to be dealt with rendered bile salt preparations of no great advantage as selective media for isolating plague bacilli from a mixture of organisms.

For culture work the spleen was usually taken. Cultures were also made from other material which, after microscopic examination, seemed suitable. After the surface of the spleen, or other organ, had been well seared, a portion of the interior was cut out with a sterile knife and placed on the first plate or culture tube. After this material had been rubbed over the surface two or three more plates or tubes were inoculated from it in series. The temperature of incubation was 30° C.

In addition to the cultural investigation, the specimens in nearly every case were tested by inoculation upon white rats or guinea-pigs or both. Animals were also inoculated, when thought necessary, with cultures or with such material from the first experimental animals as required further investigation. The inoculation was usually made subcutaneously, but in some cases cutaneously.

In the case of the specimens sent from Ipswich for diagnosis the

spleen and liver were usually selected for inoculation. After the surfaces of these organs had been thoroughly seared, portions of the interior were transferred to a small test tube, well rubbed up with a glass rod and then emulsified with normal saline. Other material, such as lymphatic glands, pleural effusion or blood, were also used when thought requisite.

No case was diagnosed as positive until a typical plague culture and typical plague infection of an inoculated animal had been obtained. With the exception of 12 cases where animal inoculation was not considered necessary, no case was settled as negative until it was proved that the material was incapable of producing plague in an experimental animal.

Enquiry was also made into the action of the plague bacilli, after isolation in pure culture, upon various carbohydrates and allied substances and into their virulence, in measured dosage of culture, for experimental animals. Broth cultures showing typical stalactites were also obtained with several of the viruses.

Results.

Out of the 15,332 rats which were dissected at the Ipswich laboratory by Drs Macalister and Brooks, specimens from 151 were sent to the Board's laboratory for diagnosis. They were there examined by the methods described above.

Thirty-five of the rats were found to be plague infected, the remaining 116 being proved negative as regards plague.

ANALYSIS OF RESULTS IN POSITIVE CASES.

Preliminary Data.

Out of the 35 rats proved to be plague infected, 21 had been caught and killed, the remaining 14 had been found dead.

At the time of the post-mortem examination the condition, as regards preservation, of 30 of the above rats was described by Drs Macalister and Brooks as either "good" or "fair"; the remaining five were either "bad" or "putrid."

Accompanying the selected material submitted to the Board's laboratory for diagnosis, full details were sent of the post-mortem and microscopic appearances which Drs Macalister and Brooks found.

These data may be classified as follows:

Group I.—In this group, to which 28 of the positive cases belong, the appearances were either typical of plague or very strongly suggestive

of it. The predominant features were marked subcutaneous congestion and general distribution in the tissues of bacilli, usually very numerous, which were morphologically identical with *B. pestis*. A good example is Rat 2876, which was described in the following detail: *Macroscopically*: The right inguinal gland was the size of a pea, with a necrotic centre and thick walls; the pelvic glands were enlarged; subcutaneous congestion was intense in the region of the right inguinal gland and was very well marked all over the trunk; no haemorrhages were seen; there was some clear pleural effusion; the liver was large, firm, generally pale, mottled, and showed numerous punctate necrotic foci; the spleen was large and firm, with well-marked granulation. *Microscopically*: A few bacilli, with some involution forms, were found in the necrotic centre and in the walls of the right inguinal gland; some bipolar bacilli were seen in the right pelvic gland; no organisms were found in the pleural effusion; scanty bipolar bacilli were found in the liver and spleen; a few plague-like bacilli were found in the heart's blood.

This case proved to be, in most respects, typical of the group. But in the remaining 27, though glandular enlargement and congestion were usually found, necrosis of a lymph gland only occurred once, and plague-like bacilli were numerous in the organs in 22 cases. Other differences which occurred in some of these 27 cases were minor and were not sufficiently marked to justify classification in a separate group.

Twelve of these 28 cases were rats which had been found dead; three of the rats were found at the post-mortem examination to be "bad" or "putrid."

Group II.—To this group seven positive cases belong. In general, the macroscopic and microscopic data cannot be regarded as affording more than slight suspicion of plague. Some bipolar bacilli were found in each case, but subcutaneous congestion was only marked in one case and was absent altogether in three. Rat 1030 belongs to this group. As this was the first case in the present enquiry which turned out to be positive, the full description of its post-mortem and microscopic appearance is of particular interest. *Macroscopically*: there was some enlargement of the submaxillary glands; there was neither subcutaneous congestion, haemorrhage, nor pleural effusion; the liver showed punctate, white necrotic foci; in the spleen there were remains of a white infarct. *Microscopically*: numerous bipolar bacilli were found in the liver and heart's blood. This is the only case, among the seven, in which bipolar bacilli were recorded as numerous; in one case, in which the tissues were decomposed, "mixed organisms" including bipolar bacilli were

found; in the remaining five cases bipolar bacilli were stated to be scanty.

Two of these seven cases were rats which had been found dead; two of the rats were found on post-mortem examination to be "bad" or "putrid."

Results of Culture Work.

Isolation of the plague bacillus by culture.

In 29 out of the 35 positive cases *Bacillus pestis* was obtained by direct culture of the original material, and in many cases without admixture of any contaminating organisms. Cultures were obtained from the spleen in 26 of these cases; in the remaining 3 they were obtained from the liver; additional cultures were obtained in 8 cases from livers and in 2 cases from lymphatic glands.

In the remaining 6 out of the 35 cases direct cultures from the original material failed. In three instances they were overgrown; in two other cases, where bile salt media alone were used, no plague colonies appeared; in the sixth case no culture was attempted, as the original material was very putrid.

In each of the above six cases cultures of *B. pestis* were obtained from the animals inoculated with the original material.

Characteristics of the plague cultures.

The following were the main characteristics of the cultures, which were obtained by the methods described above (p. 289).

On examination the next day, within 24 hours after inoculation on agar, plague colonies were usually visible with the aid of a hand lens as minute transparent points; when the material inoculated contained numerous plague bacilli, a thin growth over the surface could be seen by the naked eye. The colonies, transparent at first, soon became denser and then exhibited a delicate but characteristic ground-glass appearance; as the colonies grew older they became completely opaque. The typical plague colony, two to three days old, was greyish-white, with a smooth, shining surface; the centre of the colony was slightly raised, the margin thin, grey, translucent and irregular; in this margin minute colonies sometimes developed. The adhesive nature of the culture was an important characteristic, to which special attention has been called by Dr Klein. This peculiarity, which developed early when incubation took place at 30° C., was readily demonstrated by touching

the growth with the platinum needle : on gently raising the needle, the culture adhered to it and was drawn out in the form of a long thread.

In subculture on agar slants, and also in primary culture from tissues rich in plague bacilli without admixture of other organisms, the surface became covered with a continuous greyish-white layer, exhibiting the characteristic stickiness when touched with the platinum needle. Not uncommonly discrete, dense colonies of plague bacilli grew up above the rest of the surface of a pure culture.

As the cultural results obtained on agar were unequivocal and distinctive and were corroborated in every instance by the production of typical plague in an experimental animal, no further culture work was considered necessary for the purpose of diagnosis.

Incidentally, a few experiments were made on the production of stalactites in broth covered with a thin layer of butter fat and kept at room temperature. Out of the six strains, selected at random, which were tested five produced typical stalactites, the sixth failed to do so.

Fermentation tests.

For the purpose of a comparative study of plague bacilli with other organisms which are often differentiated from each other by means of fermentation tests, each of the 35 plague viruses was submitted to a series of these tests.

To ordinary peptone water, made with tap water and coloured with 10 per cent. litmus solution, .5 per cent. of one of the following chemically pure carbohydrates or other compounds was added : glucose, fructose, galactose, maltose, lactose, saccharose, raffinose, iso-dulcite, glycerin, mannite, dulcite, adonite, inulin, and salicin. Each of these media was put up in Durham's fermentation tubes. Litmus milk and malachite green peptone water were also used as test media.

With all 35 viruses acid, but no gas, was produced in—glucose, fructose, galactose, maltose, mannite, adonite, and salicin. The reactions with the last two were slow, salicin taking about 6 days and adonite about 12 days.

With all 35 viruses no change of reaction was produced in—lactose, saccharose, raffinose, dulcite, glycerin, inulin, and litmus milk. The period of observation, in each case, was 28 days. During this period no appreciable change was noted in the malachite green medium, but after about 5 weeks' incubation many strains slightly decolourised this medium.

With 4 of the viruses acid was formed, in 20 days, with iso-dulcitate ; with the other 31 viruses during this period no change was produced with the compound, but in the case of two of these 31 acid was formed after a further incubation of 20 days.

For the purpose of control, all the above tests were performed also with two previously authenticated strains of plague bacilli ; for one of these we are indebted to Dr Klein, for the other to Dr Rowland, of the Lister Institute. These two strains produced no change in iso-dulcitate ; in all the other tests they corresponded exactly to the 35 strains mentioned above.

Results of Animal Experiments.

Inoculation of rats with plague-infected tissues.

With the exception of a few cases where the cutaneous method of inoculation was adopted, the material was inoculated subcutaneously in the right groin.

The day after inoculation the rats usually looked ill, with staring coat, and local tenderness and swelling could be felt on palpation. They rapidly became worse, dyspnoea being marked, and died in from 2 to 8 days ; death usually occurred between the second and the fourth day. In every case subcutaneous inoculation produced a fatal result, a tumour being formed at the site of inoculation.

The local lesion was invariably found to be necrotic and oedematous. It was surrounded by an area of congestion which was often intense. There were general redness and vascular engorgement throughout the ventral subcutaneous tissues. These features were constant and particularly conspicuous.

The inguinal and iliac glands were usually enlarged and often congested and necrosed, the extent of the lesions being proportionate to the duration of the animal's life. The axillary and submaxillary glands were sometimes enlarged and congested.

In several cases the spleen did not appear abnormal in size, colour, or consistency. In others it was enlarged, firm, and dry on section. In one rat, which lived for 8 days, the spleen was peppered with grey foci.

In animals which died in from 2 to 3 days the liver generally showed nothing more than congestion. In later cases it showed either small, irregular patches of necrosis or minute irregular grey foci.

TABULAR STATEMENT OF RESULTS.
Details of Cultural Results and Animal Inoculations in the Diagnosis of the 35 Positive Cases.

* The Roman numerals refer to the grouping on pp. 291 and 292.

Number of Rats	Condition	Results of cultures from original material, as regards growth of plague bacilli	Results of animal experiments			
			Material inoculated	Animals	Duration of life	Post-mortem results
1030	II	Discrete colonies from liver on mannite bile salt	Liver	Rat 30	Died, 3 days	Acute plague. Bacilli numerous in liver, spleen, and blood.
			Spleen	G.P. 507	Died, 5 days	Acute plague. Bacilli abundant in spleen.
1264	II (<i>putrid</i>)	Cultures, on bile salt media, failed	Liver	Rat 31	Died, 4 days	Acute plague. Bacilli numerous in spleen.
			Liver	Rat 32	Died, 6 days	Acute plague. Bacilli numerous in liver, spleen, and blood. Cultures from Rats 31 and 32 typical of plague.
1691	I (<i>found dead</i>)	Cultures, on bile salt media, failed	Liver	Rat 36	Died, 3 days	Acute plague. Bacilli numerous in spleen and liver.
			Liver	Rat 37	Died, 3 days	Acute plague. Bacilli (not numerous) in spleen and liver.
1694	I (<i>found dead</i>)	Discrete colonies from liver on lactose bile salt	Spleen	G.P. 514	Died, 8 days	Typical plague. Bacilli fairly numerous in spleen. Cultures from Rat 37 typical of plague.
			Liver	Rat 38	Died, 3 days	Acute plague. Bacilli fairly numerous in liver and a few in spleen.
			Liver	Rat 39	Died, 3 days	Acute plague. Bacilli numerous in liver and spleen.
1769	I	Discrete colonies from liver and spleen on mannite bile salt	Spleen	G.P. 515	Killed, 16 days	Healthy. Cultures from Rat 38 typical of plague.
			Liver	Rat 40	Died, 2 days	Neerotic local lesion; much oedema, spreading over thoracic wall; nothing definite in organs. Two bipolar bacilli seen in spleen, none in liver.
			Pleural fluid	Rat 41	Died, 6 days	Typical plague. A few bacilli in spleen, fairly numerous in liver.
			Spleen	G.P. 540	Died, 6 days	Typical plague. Bacilli numerous in spleen.
2064	II	Pure culture from spleen on agar slants	Liver	Rat 51	Died, 4 days	Cultures from Rat 40 typical of plague.
			Liver	G.P. 561	Died, 4 days	Acute plague. Bacilli rather scanty in spleen. Acute plague. Bacilli abundant in spleen.

2156	II (<i>found dead</i>)	Pure cultures from spleen and liver on agar slants	Liver	Rat 52	Died, 5 days	Acute plague. A few bacilli in spleen.
			Liver	Rat 53	Died, 3 days	Acute plague. Bacilli rather scanty in spleen.
			Liver	G.P. 566	Died, 6 days	Acute plague. Bacilli abundant in spleen.
2522	II (<i>putrid - found dead</i>)	No culture attempted	Spleen and Liver	Rat 72 (<i>cutaneous</i>)	Killed, 13 days	Healthy.
			Spleen and Liver	Rat 73 (<i>cutaneous</i>)	Died, 6 days	Acute plague. Bacilli scanty in spleen, many in axillary gland.
2576	I (<i>found dead</i>)	Discrete colonies from inguinal bubo on maumite bile salt slants. Abundant colonies on agar slants from spleen	Spleen	Rat 94	Died, 3 days	Cultures from Rat 73 typical of plague.
			Liver	G.P. 652	Died, 3 days	Acute plague. Bacilli moderately numerous in spleen.
3346	I (<i>found dead</i>)	Overgrown (liver and spleen)...	Spleen and Liver	Rat 116	Died, 3 days	Acute plague. Bacilli abundant in spleen.
			Spleen and Liver	Rat 117	Died, 4 days	Acute plague. Bacilli moderately numerous in spleen.
						Soft caseous nodule at site of inoculation, without congestion; spleen mottled with greyish necrotic patches; two similar patches in liver. A few bipolar bacilli in spleen.
3430	I (<i>found dead</i>)	Pure cultures from spleen on agar slants; cultures almost pure from liver on same medium	Spleen and Liver	Rat 118	Died, 2 days	Cultures from liver and spleen of Rat 116 typical of plague. Cultures from spleen of Rat 117 overgrown with coliform growth.
			Spleen and Liver	Rat 119	Died, 3 days	Acute plague. Bacilli moderately numerous in spleen.
4849	I	Numerous colonies in pure culture from spleen and almost pure from liver—on agar slants	Spleen and Liver	Rat 143	Died, 5 days	Acute plague. No bacilli seen in spleen, fairly numerous in iliac gland.
			Spleen and Liver	G.P. 706	Died, 10 days	Pure cultures from spleen and liver.
5381	I	Pure culture from spleen on agar slants	Spleen and Liver	Rat 144	Died, 8 days	Acute plague. Bacilli moderately numerous in spleen and axillary gland.
			Spleen and Liver	G.P. 743	Died, 6 days	Plague lesions well marked. Bacilli in spleen.
						Purulent sinus at site of inoculation, slight injection of subcutaneous vessels; right inguinal gland enlarged; liver and spleen peppered with irregular grey foci. No bipolar bacilli seen in spleen; two seen in liver.
						Coliform colonies from spleen but no plague colonies.
						Acute plague. Bacilli abundant in spleen.

TABULAR STATEMENT OF RESULTS (continued).

Number and Condition of Rats		Results of cultures from original material, as regards growth of plague bacilli	Results of animal experiments			
Number	Condition		Material inoculated	Animals	Duration of life	Post-mortem results
5382	I (found dead)	Discrete colonies from liver on one agar slant. Three other tubes overgrown	Liver	G. P. 744	Died, 4 days	Acute plague. Bacilli abundant in spleen. Pure culture isolated from spleen.
7643	I	Abundant growth on agar slants from spleen and liver, almost pure	Spleen and Liver	Rat 157	Died, 2 days	Acute plague. Bacilli moderately numerous in spleen, from which a pure culture was obtained.
8086	I (found dead)	Discrete colonies on agar slants from spleen	Spleen and Liver	Rat 160	Died, 3 days	Acute plague. Bacilli rare in spleen, moderately numerous in iliac gland.
10169	I	Pure culture on agar slants from spleen	Spleen and Liver	Rat 169	Died, 2 days	Acute plague. Bacilli moderately numerous in spleen, abundant in iliac gland.
10840	I (found dead)	Pure culture on agar slants from spleen	Spleen and Liver	Rat 175	Died, 2 days	Necrosis at site of inoculation, with oedema but no congestion; slight enlargement of iliac gland; spleen firm; liver lobules outlined. Numerous bacilli in iliac gland.
10848	I	Discrete colonies on agar slants from spleen and liver	Spleen and Liver	Rat 176	Died, 4 days	Acute plague. Bacilli scanty in spleen, moderately numerous in iliac gland. Pure culture obtained from spleen.
11407	II (bad)	Discrete colonies on agar slants from spleen; contaminating organisms numerous	Spleen and Liver	Rat 180	Died, 4 days	Oedematous necrotic tissue at seat of inoculation, without congestion; iliac gland enlarged; spleen firm; liver peppered with irregular grey foci. One doubtful bacillus seen in spleen. Cultures from liver and spleen overgrown. G. P. 876, inoculated from the liver, died in 5 days from acute plague.
11724	I (found dead)	Pure cultures on agar slants from spleen	Spleen and Liver	Rat 184	Died, 4 days	Acute plague. No bacilli seen in spleen, but pure culture was obtained from it.
12012	I	Abundant, pure culture on agar slants from spleen	Spleen and Liver	Rat 189	Died, 3 days	Acute plague. Abundant bacilli in iliac gland.
12013	I	Abundant, pure culture on agar slants from spleen	Spleen and Liver	Rat 190	Died, 3 days	Acute plague. Abundant bacilli in iliac gland.
12245	I	Overgrown ...	Spleen and Liver	Rat 192	Died, 3 days	Very marked local congestion, necrosis, and oedema: organs apparently normal. Bacilli scanty in spleen, abundant in local lesion. Culture isolated from spleen. Rat 197, fed with organs of Rat 192, died in 5 days from plague, with submaxillary bubo and abundant bacilli in spleen.

13663	I (<i>found dead</i>)	Pure culture on agar slants from spleen	Spleen and Liver	Rat 199	Died, 3 days	Acute plague. Bacilli abundant in iliac gland and numerous in spleen.
13712	I	Pure culture on agar slants from spleen	Spleen and Liver	Rat 200	Died, 2 days	Highly congested, necrotic local lesion; slight general subcutaneous congestion; iliac gland slightly enlarged; spleen small and rather firm. Bacilli abundant in iliac gland, a few in spleen.
13713	I	Confluent pure culture on agar slants from spleen	Spleen and Liver	Rat 201	Died, 3 days	Acute plague. Bacilli moderately numerous in spleen and abundant in iliac gland. Culture obtained from spleen.
13817	I	Confluent pure culture on agar slants from spleen	Spleen	Rat 202	Died, 2 days	Acute plague. Bacilli abundant in iliac gland and spleen.
14499	I	On agar slants pure confluent growth from lymphatic gland, discrete colonies from spleen and liver	Spleen and Liver Gland	Rat 209 G.P. 897	Died, 3 days Died, 6 days	Acute plague. Bacilli moderately numerous in spleen, abundant in iliac gland.
14816	II	Discrete colonies from spleen on agar slants	Spleen	Rat 220	Died, 4 days	Acute plague. Bacilli abundant in spleen.
14854	I	Pure cultures from liver and spleen on agar slants	Spleen	Rat 221	Died, 3 days	Slightly congested necrotic local lesion; nothing else abnormal. No bacilli seen in smear from spleen. Pure culture of plague from spleen.
14855	I	Discrete colonies from spleen on agar slants	Spleen	G.P. 902	Died, 5 days	Acute plague. Bacilli moderately numerous in spleen.
14911	I	Pure cultures on agar slants from liver and spleen	Spleen	G.P. 909	Died, 4 days	Acute plague. Bacilli abundant in spleen.
15079	I (<i>found dead</i>)	Discrete colonies from spleen on agar slants	Spleen and Liver	Rat 230	Died, 4 days	Purulent nodule at site of inoculation; general subcutaneous congestion; necrotic foci in iliac gland. Two bacilli found in smear from iliac gland.
15219	I	Discrete colonies from spleen on agar slants	Spleen	Rat 229	Died, 2 days	Acute plague. Bacilli moderately numerous in inguinal gland.

The lungs were frequently oedematous and congested, but no pleural effusion was found.

Plague bacilli were almost invariably numerous in the local lesion and the nearest glands, being usually irregular in size and shape and often showing involution forms. In the spleen they were often scanty, especially when the animal died early with marked local reaction. In some cases the bacilli were abundantly disseminated throughout the body and in the blood.

Inoculation of guinea-pigs with plague-infected tissues.

The material was inoculated subcutaneously in the right groin, in the same manner as with the rats.

The day after inoculation it was usually found that the animals were quiet and that local tenderness and some swelling could be detected on palpation. Day by day the animals rapidly became more ill and lost flesh, whilst the local swelling increased in size. At death there was usually a firm, prominent swelling and the right hind leg was drawn up. Death occurred between the third and the tenth day; in the majority of cases it was between the fifth and the sixth day.

At the site of inoculation there was found in the subcutaneous tissue an infiltrating tumour which, on section, was seen to be centrally necrotic and to be surrounded by an area of intense congestion and oedema. The size of the tumour varied, being usually larger in the animals which lived longer; the largest was the size of a pigeon's egg and was found in a guinea-pig which had lived for ten days after inoculation.

Subcutaneous congestion and oedema extended for a considerable distance beyond the local lesion, with engorgement of the subcutaneous capillaries and veins; but the oedema was never found to spread completely over the ventral subcutaneous tissues.

The inguinal and iliac lymphatic glands were enlarged and congested, particularly in the cortex; their interior usually showed extensive necrosis.

The spleen was usually enlarged, congested, and either mottled with pale, irregular necrotic areas or studded with discrete, soft, grey nodules ranging in size up to 1 mm. in diameter. In cases of very acute infection ending fatally in about three days the spleen remained small and did not exhibit macroscopic lesions.

The liver, as a rule, was enlarged; sometimes it showed nodules similar to those found in the spleen; in other cases it was peppered with

irregular, grey foci. In cases which terminated fatally in three days no lesions were visible.

The lungs were usually congested and oedematous, sometimes with patches of consolidation. They were frequently studded with soft, greyish-white nodules with a congested periphery. The largest of these nodules were found in the less rapidly fatal cases; in one animal, which had lived for ten days, they attained the diameter of 5-6 mm. Pleural effusion was found sometimes.

In almost every instance the tissues of the animals contained enormous numbers of plague bacilli; in smears from the spleen or the lung nodules this feature was particularly noticeable.

Inoculation of rats and guinea-pigs with plague cultures.

In rats inoculated with culture, the oedema, congestion, and necrosis of the local lesion, the general subcutaneous congestion, and the enlargement, congestion, and necrosis of the inguinal and iliac glands were all features which corresponded closely with the results obtained by the inoculations of infected tissues. As compared with tissue emulsions, pure cultures produced less macroscopic change in the spleen and liver; the spleen, though sometimes enlarged and often firm and dark on section, did not show foci of necrosis; the liver was generally normal in appearance, though occasionally showing a few grey foci. The lungs were generally oedematous and sometimes congested; in a small proportion of the cases (less than a quarter) there was a little pleural exudate.

In guinea-pigs culture inoculations produced very much the same effects as emulsions of tissues.

The Virulence of the Plague Strains.

In the earlier part of the enquiry the cultures isolated from each case of rat plague were tested for virulence and were compared in this respect with two previously authenticated strains of plague bacilli received respectively from Dr Klein and from Dr Rowland at the Elstree Laboratory. It was found that pure cultures of all the strains were of high, and of about equally high, virulence for rats and guinea-pigs. It was not considered necessary to continue these culture tests systematically throughout the enquiry because in no instance was a virus obtained which, when inoculated in the form of a tissue emulsion, afforded any indication of being lower in virulence than the viruses already tested by inoculation both of cultures and of tissue emulsions.

The estimation of dosage was made by weighing on a chemical balance a platinum loop charged with culture and then subtracting the weight, previously ascertained, of the platinum loop. The weighed culture was finely emulsified with the requisite amount of normal saline solution to afford a convenient volumetric measure of dosage.

Most of the inoculations were made subcutaneously, but virulence for rats was also tested by the cutaneous method, one or two loopfuls of dilute emulsion of culture being smeared over the shaved and lightly scarified skin at the root of the tail.

The results of these virulence tests are tabulated below.

Virulence of the Plague Cultures for Rats inoculated subcutaneously.

Number or name of virus	Dose inoculated, in mgms.	Number of rat inoculated	Duration of life, in days
1264	·1	46	3
1030	·1	48	5
1694	·01	42	3
1691	·01	44	3
1769	·01	57	5
2064	·01	58	4
"Klein"	·01	60	3
"Elstree"	·001	62	4
2156	·001	131	3
3346	·001	133	3
3430	·001	135	4
4849	·0001	150	[remained healthy]
4849	·00001	151	5

Virulence of the Plague Cultures for Guinea-pigs inoculated subcutaneously.

Number or name of virus	Dose inoculated, in mgms.	Number of guinea-pig inoculated	Duration of life, in days
1030	1	559	4
1030	·1	560	5
1694	·1	554	5
1691	·1	556	6
1264	·1	557	6
1769	·1	591	3
2064	·1	593	5
"Klein"	·1	595	5
1691	·01	555	5
1264	·01	558	4
1769	·01	592	5
2064	·01	594	3
"Klein"	·01	596	7
"Elstree"	·01	597	4
"Elstree"	·001	598	5
2156	·001	689	13
3346	·001	690	8
3430	·001	692	8
4849	·0001	760	[killed, 18 days ; local disease only]
4849	·00001	761	8

Virulence of the Plague Cultures for Rats inoculated cutaneously.

Number or name of virus	Number of loopfuls of dilute culture emulsion used	Number of rat	Duration of life, in days
1694	1	43	6
1691	1	45	[killed, 15 days; healthy]
1264	2	47	4
1030	?	50	5
1769	1	56	4
2064	1	59	3
"Klein"	1	61	4
"Elstree"	1	63	4
2156	1	132	5
3346	1	134	3
3430	1	136	5
5381	2	152	4
11407	?	174	[killed, 24 days; healthy]
14816	?	231	3

ANALYSIS OF RESULTS IN NEGATIVE CASES.

Preliminary Data.

Out of the 116 cases which were under more or less definite suspicion at the post-mortem examination but proved negative when further investigation was made at the Board's laboratory, 105 had been caught and killed; the remaining 11 had been found dead.

At the time of the post-mortem examination the condition, as regards preservation, of 93 of the above rats was described by Drs Macalister and Brooks as either "good" or "fair"; the remaining 23 were either "bad" or "putrid."

The post-mortem and microscopic details furnished from the Ipswich laboratory may be classified as follows:

Group I.—Five cases fall within this group and resemble the positive cases found in Group I (p. 291) in so far as their descriptions are strongly suggestive of plague. Subcutaneous congestion was present in all and was very marked in one. In four cases bipolar bacilli were numerous and generally distributed; in the fifth they were generally distributed in small numbers. With the exception of one liver, necrotic or granular changes are described in the livers and spleens of all the cases. Pleural effusion was found in every case and glandular enlargement in all but one. One rat (4843) was in bad condition and one (10,510) was found dead.

TABULAR STATEMENT.

Details of the 33 Negative Cases where Lesions were produced in Experimental Animals.

* The Roman numerals refer to the grouping on pp. 303, 307. † "*B. Gärtner*," throughout this table, means a bacillus belonging to the Gärtner group.

Number and Condition of Rats		Results of animal experiments	
Number	*Condition	Animals	Results
544	III	G.P. 501	Died, 6 days; only slight local reaction, necrotic foci in liver, spleen apparently normal. <i>B. coli</i> isolated from spleen.
711	II	G.P. 504 Rat 27	Died, 2 days; septic cellulitis. † <i>B. Gärtner</i> isolated from blood and spleen. Killed, 5 days; necrosis at site of inoculation, spleen peppered with minute, irregular, grey foci. <i>B. Gärtner</i> isolated.
1444	III	G.P. 513 Rat 33	Died, 1 day; acute infection. <i>B. coli</i> isolated from inguinal gland. Killed, 7 days; like Rat 27. <i>B. Gärtner</i> isolated from spleen.
2353	II	G.P. 611 Rat 66 (<i>cutaneous</i>) Rat 67	Died, 3 days; necrotic local lesion and subcutaneous congestion. <i>B. coli</i> isolated. Killed, 6 days; healthy. Killed, 6 days; healthy.
2801	II (<i>putrid</i>)	G.P. 656 G.P. 657	Died, 1 day; septic cellulitis. Culture of colon type isolated from spleen. Died, 3 days; septic cellulitis.
3023	II	G.P. 658 G.P. 659	Rat 113 was inoculated cutaneously with culture isolated from original material. Died, 7 days; grey foci in liver. <i>B. Gärtner</i> isolated from liver. Died, 1 day; intense oedema and subcutaneous congestion. Culture isolated was inoculated into 2 guinea-pigs which died in 1 and 3 days from acute disease unlike plague, and into 2 rats which remained healthy. Died, 2 days; intense oedema and subcutaneous congestion. From each G.P. a culture similar to the one from original material was isolated.

3030	II	Discrete colonies from spleen. A coccus isolated	Rat 106	Killed, 8 days: no local lesion, grey foci in liver and spleen. <i>B. Gärtner</i> isolated.
3051	II	Discrete colonies from spleen. A coccus isolated	Rat 108	Killed, 8 days; purulent nodule at site of inoculation, grey foci in liver and spleen. <i>B. Gärtner</i> isolated.
3052	II	Discrete colonies from spleen and liver. A coccus isolated	Rat 109	Killed, 8 days; grey foci in liver and spleen. <i>B. Gärtner</i> isolated.
3053	II	Discrete colonies. A coccus isolated	Rat 110	Ditto ditto ditto.
3056	III (<i>putrid</i>)	Discrete colonies from liver. A coccus isolated	Rat 111	Ditto ditto ditto.
3138	II	Discrete colonies from spleen and liver	Rat 112	Died, 9 days; no lesions beyond slight local congestion at site of inoculation; colon-like bacillus isolated from liver.
3495	III	Discrete colonies from liver	Rat 126	Died, 2 days; slight local congestion only. Some bipolar bacilli in spleen, from which colon-like culture was obtained.
3615	III	Discrete colonies from gland at root of penis, yielding <i>B. coli</i>	Rat 127	Died, 3 days; no local reaction, grey foci in spleen. <i>B. Gärtner</i> isolated.
3649	II	Discrete colonies from gland at root of penis. A coccus isolated	Rat 128	Killed, 13 days; local abscess, spleen speckled with grey foci. <i>B. Gärtner</i> isolated.
3762	III	Overgrown	G.P. 687	Died, 2 days; septic cellulitis; microscopically, no plague-like bacilli.
4187	III	Overgrown	G.P. 697	Killed, 13 days; normal except 2 yellow foci in spleen. Motile bacilli isolated from spleen.
4242	II	Discrete colonies from liver	G.P. 696 G.P. 695	Died, 3 days; acute cellulitis; no organisms found in spleen. Killed, 13 days; healthy.
4659	II (<i>putrid</i>)	Discrete colonies from spleen	Rat 141	Died, 2 days; subcutaneous oedema, necrosis and congestion. Bipolar bacilli found in spleen and iliac gland. Saccharose fermenter, isolated from spleen, inoculated in doses of 1 mg. into Rat 149 and G.P. 759; both animals killed, 18 days,—healthy.
6427	II (<i>putrid</i>)	Discrete colonies from spleen	G.P. 770	Died, 8 days; small abscess only.
8284	II	Discrete colonies from spleen	Rat 163†	Killed, 12 days; no local lesion; grey foci in liver and spleen; no bipolar bacilli in spleen.

Number and Condition of Rats		Results of animal experiments		
Number	Condition	Results of cultures from original material	Animals	Results
8439	II (found dead)	Discrete colonies from spleen	Rat 162	Died, 2 days; slight necrosis at site of inoculation, many bipolar bacilli; a few in spleen. Colon-like colonies were isolated from local lesion and spleen of Rat 162. Rat 166, inoculated from local lesion of Rat 162, was killed in 9 days and found healthy.
10150	I (found dead)	Overgrown	Rat 168	Died, 4 days; local necrosis and oedema, a few grey foci in liver; coliform organism isolated.
10549	II	Discrete colonies from liver	Rat 173	Killed, 7 days; no local lesion; grey foci in liver and spleen; no bipolar bacilli in spleen.
10678	III	Discrete colonies from spleen and liver	Rat 174	Died, 7 days; slight necrosis, but no congestion at site of inoculation; grey foci in liver and spleen. <i>B. Gärtner</i> isolated.
12194	II (bad)	Overgrown	Rat 191	Died, 6 days; local abscess, few grey foci in liver. <i>B. Gärtner</i> isolated.
12520	III	Discrete colonies from spleen	Rat 193	Killed, 8 days; slight local reaction; grey foci in liver and spleen. <i>B. Gärtner</i> isolated.
12919	III (bad)	Discrete colonies from spleen	Rat 195	Killed, 7 days; slight local reaction; grey foci in liver and spleen. <i>B. Gärtner</i> isolated.
14638	III (bad)	Discrete colonies from spleen	Rat 214	Killed, 8 days; no local reaction; grey foci in spleen and liver.
14790	II	Discrete colonies from spleen, yielding <i>B. Gärtner</i>	Rat 217	Died, 6 days; local necrotic nodule, grey foci in liver and spleen; colon-like culture from spleen.
14792	II	Overgrown	Rat 218	Died, 7 days; necrosis at site of inoculation, congestion without oedema; culture from spleen unlike plague.
14793	II	Overgrown	Rat 219	Died, 5 days; small necrotic nodule at site of inoculation, no subcutaneous congestion, spleen firm, irregular grey foci in liver. Culture from liver coliform.
15064	III	Discrete colonies from spleen	Rat 224	Killed, 7 days; no local lesion, grey foci in liver and spleen.

Group II.—Sixty-one of the negative cases fall into this group. They resemble the positive cases in Group II (p. 292) in that the macroscopic and microscopic data afford slight, but no more than slight, suspicion of plague. The following are the main general facts concerning these 61 cases :

Macroscopically :—Subcutaneous congestion was present in 39 cases but absent in 22 ; pleural effusion was present in 42 but absent in 19 ; mottling or necrotic foci were present in 30 livers but absent in 31 ; granulation or necrotic foci were present in 26 spleens but absent in 35 ; enlargement of lymphatic glands was seen in the majority of cases.

Microscopically :—In each case bipolar bacilli were found in some part of the body, though usually they were not numerous ; in 54 cases the liver or spleen, or both organs, were examined, and in 53 of these cases bipolar bacilli were found in one or both of these organs.

In eight cases there were found—subcutaneous congestion, bipolar bacilli in the liver and spleen, pleural effusion, and the above-mentioned changes in the liver and spleen.

Eight of the 61 rats had been found dead ; 13 were “ bad ” or “ putrid ” when dissected.

Group III.—This group comprises 50 cases, each presenting some element of doubt at the time of the post-mortem examination, though it was obvious that further investigation was needed before plague infection could be proved or even definitely suspected.

The following are the main features of these cases :

Macroscopically :—Subcutaneous congestion was present in 18 but absent in 32 ; pleural effusion was present in 21 but absent in 29 ; liver changes were present in 25 but absent in 25 ; spleen abnormalities were present in 21 but absent in 29 ; enlargement of lymphatic glands was noted in all but 12.

Microscopically :—Bipolar bacilli were only found once in the liver or spleen ; they were present in other regions of the body in 33 cases ; in 16 cases no bipolar bacilli were found in any part of the body.

Two of the above 50 cases had been found dead ; 9 were “ bad ” or “ putrid ” when dissected.

Results of Cultures and Animal Experiments.

Cultures were usually made from the spleen ; under special circumstances the liver, a lymphatic gland, pleural exudate, or other material was also used.

Cultures were attempted from the original material of all except four of the cases which turned out to be negative, these four being omitted because the material was too putrid to give any prospect of success. In 20 cases the agar media inoculated remained sterile; in 16 the primary cultures were overgrown, so that it was impossible to decide whether or no they contained plague bacilli; and in 76 discrete colonies were obtained, which rendered it possible to decide, either directly or after subculture of any doubtful colonies, that no growth of plague bacilli had been obtained.

The animals inoculated with original material remained healthy in 71 out of the 116 negative cases, and after being kept alive for a sufficient period (from one to two weeks) were killed, and proved by post-mortem examination to be free from disease.

In 12 cases it was not thought necessary to inoculate animals with original material, and in these the microscopic and cultural evidence appeared conclusive. In 2 of these cases the culture media inoculated remained sterile; in 8 discrete colonies, unlike plague, were obtained; and in 1, though the agar cultures were overgrown, cultures on bile salt media yielded discrete colonies but failed to develop any resembling plague.

In the twelfth case, Rat 818, a culture from the submaxillary gland, though more luxuriant than plague, was somewhat sticky in character; it was therefore tested on a guinea-pig and a rat, but failed to produce any disease. It was also observed that the culture fermented raffinose and saccharose.

In 33 cases the original material inoculated produced, in one or more of the animals, lesions, sometimes fatal, which were distinguished from plague either directly by macroscopic and microscopic examination or after further investigation, including the raising of cultures and, where necessary, animal inoculation.

Bacteria of some Special Interest.

Bacilli belonging to the Gärtner group.

An organism belonging to this group was isolated sixteen times, thrice from the original material and in thirteen instances from rats inoculated with original material. A feature of these cases was the occurrence in the tissues of bipolar staining bacilli closely resembling the plague bacillus.

The organisms gave the following reactions. Acid and gas were produced in glucose, galactose, fructose, mannite, and dulcitol. In maltose acid was produced, sometimes with and sometimes without gas. In glycerin and iso-dulcitol acid was produced but no gas. No change of reaction occurred in lactose, saccharose, raffinose, inulin, or salicin. Litmus milk became acid at first, but afterwards changed to alkaline. The above test media were kept under observation in every instance for at least 18 days.

Several other cases, in addition to the above 16, produced in experimental rats lesions typical of Gärtner infection, but it was not thought necessary to investigate these culturally.

A cocco-bacillus from a rabbit.

In addition to the rats' tissues, specimens from one rabbit were received for examination as being under suspicion of plague.

The animal had been found dead. The post-mortem report stated that there was subcutaneous congestion, a small and granular spleen, a congested liver with necrotic patches, and copious pleural effusion; numerous bipolar bacilli were seen in the liver and the heart's blood, and a few in the spleen.

From the tissues forwarded for examination, the liver and spleen yielded on agar slants pure cultures of a cocco-bacillus; the colonies presented a resemblance to those of plague in their general appearance, but were more translucent; the cultures grew more readily than those of plague and were not sticky.

Rat 212 was killed 13 days after inoculation with an emulsion of the liver of the rabbit. There was an ulcer at the site of inoculation, but no glandular enlargement; with the exception of a few areas of congestion in the lungs, the organs were normal.

G.P. 900 died one day after inoculation with an emulsion of the rabbit's spleen. The local lesion was deeply congested and oedematous, and there was oedema of the adjacent subcutaneous tissue. There was slight excess of peritoneal fluid, but all the internal organs appeared normal. Swarms of very small bipolar bacilli were found in the local lesion, and a few short, thick bacilli in the spleen and blood. A culture from the local lesion proved identical with those obtained from the tissues of the original rabbit.

Rabbit 13 died 3 days after cutaneous inoculation in the ear with a minute dose of culture from the original rabbit's spleen. Post-

mortem, there were some thickening and bluish redness of the ear ; the spleen and liver were both covered with a thin fibrinous layer which was easily removed ; irregular, greyish foci were then found in the substance of each organ : the lungs were congested and oedematous. In the spleen small bacilli were abundant ; in the blood small bacilli with well-marked bipolar staining were numerous.

The cultural characteristics of the organism were as follows : it was non-motile, Gram-negative, and formed a deposit in peptone water without producing turbidity. It formed acid, but no gas, in media containing respectively--glucose, galactose, fructose, saccharose, mannite, and glycerin. It produced no change of reaction in media containing maltose, lactose, raffinose, iso-dulcitol, dulcitol, adonitol, salicin, or inulin : no change was produced in litmus milk or malachite green.

From the above cultural and animal tests, the organism was evidently the bacillus of rabbit septicaemia.

Colonies bearing some resemblance to plague.

In 19 of the cases which proved to be negative, the primary cultures contained colonies which, during the first two days, bore some resemblance to plague colonies. They became visible within the first twenty-four hours after inoculation as small, almost transparent points. Subsequently their development was slow ; they remained small and transparent, but gradually became slightly raised in the centre. Subcultures grew in the form of discrete colonies, and never produced more than a very thin and transparent layer. After the first two days, therefore, there was no difficulty in distinguishing these growths from plague. As a matter of subsidiary interest, however, they received further investigation.

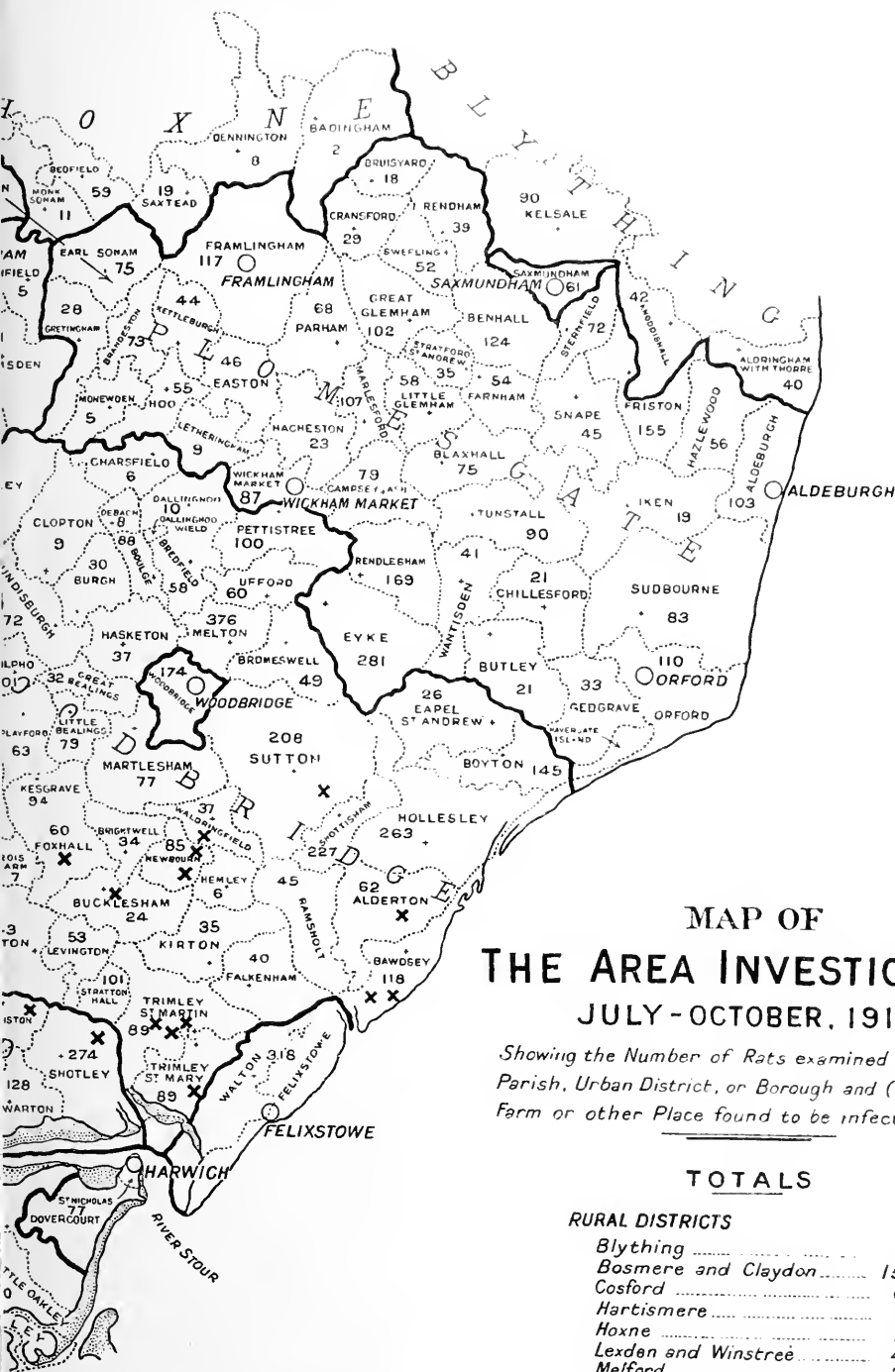
In every case examined the colonies consisted of a Gram-positive coccus with a tendency to assume slightly elongated forms which might perhaps be called very short bacilli.

The organism, when tested after being in culture for a considerable period, was not pathogenic for mice, no more than a small, purulent focus being formed at the site of inoculation. One strain inoculated subcutaneously into a rat and a guinea-pig failed to produce any disease.

In seven cases the coccus produced acid and clot in litmus milk, and acid, without gas, in glucose, galactose, fructose, maltose, saccharose, lactose, mannite, dextrin, and salicin. No change of reaction was obtained in raffinose, dulcitol, or inulin. In five other cases the organism was a somewhat less active fermenter, saccharose being unattacked in

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MAP OF THE AREA INVESTIGATED JULY-OCTOBER, 1911.

Showing the Number of Rats examined from each
Parish, Urban District, or Borough and (X) each
Farm or other Place found to be infected

TOTALS

RURAL DISTRICTS

Blything	172
Bosmere and Claydon	1519
Cosford	648
Hartismere	5
Hoxne	178
Lexden and Winstree	475
Melford	283
Plomesgate	2718
Samford	2398
Tendring	764
Woodbridge	3585

URBAN, &c.

Colchester	359
Felixstowe and Walton	318
Hadleigh	109
Harwich and Dovercourt	77
Ipswich	1489
Saxmundham	61
Woodbridge	174

15,332

one case, mannite and salicin in a second, mannite and lactose in a third, and mannite, lactose and salicin in a fourth. In the fifth case the coccus had no action on litmus milk, and failed to attack lactose, saccharose, mannite and salicin.

It is doubtful whether these cocci were responsible for any pathogenic changes in the rats from which they had been obtained, and it is noteworthy, in this connection, that all the experimental rats inoculated with the original material remained healthy. They were all from cases showing marked pleural effusion, but there is no evidence that the cocci were responsible for this condition.

EVIDENCE AS TO DISTRIBUTION AND APPARENT LIMITATION OF INFECTION.

The Area from which Rats were collected.

The area investigated is shown on the accompanying map, prepared from data furnished by Mr Huddart.

At the beginning of the enquiry attention was confined to the borough of Ipswich and its environment, the rural districts of Samford and Woodbridge, and the urban districts of Woodbridge and of Felixstowe and Walton, this being the area where plague rats had previously been found.

Six rats obtained during the latter half of July from the parishes of Sutton, Bawdsey, and Trimley St Mary, in Woodbridge rural district, and from the parish of Bentley, in Samford rural district, proved to be plague infected.

As soon as these cases were fully established, steps were taken to extend the area of enquiry, whilst continuing the search for infected localities in the original area. The enlarged area comprised a larger field in East Suffolk, a small portion of West Suffolk, and a strip of Essex adjacent to the south of Suffolk. Various reasons determined the actual demarcation of the area. To the north it included certain parishes from which reports had been received in 1910 of rats being found dead in unusual numbers; to the north-west it included the whole of Bosmere and Claydon rural district, which had not been searched during the Board's enquiry in the beginning of the year 1911; to the west it extended far enough to include a parish in which a plague-infected hare had been found in 1910; and to the south it took in a fairly wide strip of land on the right bank of the Stour, including a parish where a plague-infected hare had been found in 1910.

Comparison with the Area previously investigated.

The present investigation has been especially directed to the whole of the area which was not examined during the rat enquiry conducted for the Board during the period January 16th to February 14th, 1911¹.

The area referred to consists of the rural districts of Cosford, Samford, Bosmere and Claydon, and Woodbridge, the urban districts of Hadleigh, Woodbridge, and Felixstowe and Walton, and the borough of Ipswich.

In addition to comprising this area, the present enquiry has extended a short distance to the north and to the south. (*See Map.*)

The result of the former enquiry was that no plague-infected rats were found in a wide peripheral zone surrounding the area omitted from that investigation. In the present enquiry plague-infected rats have been found only in this last-mentioned area, and only in a limited portion thereof, which corresponds closely to, but is slightly more limited than, the area proved to be infected with rat-plague before the former enquiry was instituted.

The Supply of Rats.

During July and August, the first two months of the present enquiry, rats were, as was anticipated, particularly difficult to obtain. They were dispersed in the open country amongst the standing crops and densely overgrown ditches and hedgerows; and during harvest farmers and their assistants were too busy to spare time for ratting.

There was, however, an important reason for commencing the present enquiry at the beginning of July. Rat-plague usually tends to expand from sporadic outbreaks into epizootic proportions at the beginning of autumn; therefore it was particularly desirable to locate early cases, in order that preventive measures might be directed to the foci where these were found, before the time arrived when rapid spread of the infection was likely to occur.

This anticipation was justified by results. The first rat (No. 1030) which proved to be plague-infected was caught on July 17th, and by the end of that month infected rats had been secured from five different foci. The Board at once advised the local authorities concerned, and the Board's investigators, as soon as a case was diagnosed, visited the farm or other premises where the case occurred, warned the occupier of the danger, and advised thorough and persistent rat destruction.

¹ Reports to Local Government Board on Public Health and Medical Subjects. New Series, No. 52.

This procedure was maintained throughout the enquiry, whenever fresh cases of rat-plague were detected.

The abnormally hot and dry weather experienced throughout July and August 1911 made rat-catching particularly difficult; at the same time it had the advantage of reducing to some extent the normal proliferation amongst rats, owing to their difficulties in obtaining the moisture requisite for their own subsistence and for suckling their young. Another reason for their scarcity was that a large number of farmers throughout the area investigated had been systematically killing rats throughout the year.

In September and October the area of enquiry had been extended, the harvest was over, the rats were beginning to come back to food supplies near farm buildings, and the farmers and their men had more time to interest themselves in ratting. For these reasons there was then no difficulty about obtaining a daily supply of as many rats as could be dealt with in the Ipswich laboratory; in fact, prompt measures had to be taken to reduce the inflow, which towards the end of September became excessive.

For many reasons the numbers of rats taken from each parish (*see* Map) varied considerably. Parishes differ greatly in size and in the number of farms which they contain; the rat population is very irregularly distributed; and in some districts the farmers are much more enterprising in rat destruction than in others. Special attention was paid to parishes which were under suspicion last year owing either to the discovery of rat-plague or to local reports of excessive mortality amongst rodents. In some of these parishes no plague could be discovered, although every effort was made to obtain for examination as many rats as could be found.

As soon as a positive case was diagnosed, further supplies were stopped from the parish whence the rat had been obtained, as it was the object of the enquiry to determine the limits of the infection rather than to ascertain how many cases of infection could be discovered in particular areas; and, owing to the migratory habits of rats, the discovery of one infected pocket was considered sufficient to stigmatise its environment, within a radius of several miles, as possibly plague-infected.

Although special search for dead rats was invited and the same price was paid for these as for rats which had been caught, out of the total 15,332 rats only 69 dead rats were brought in. During the September and October of the previous year (1910) dead rats were reported in large

numbers, particularly within or near to localities where the existence of rat-plague had been proved. All the known pockets where rat-plague had previously been found were re-examined several times during the present enquiry, but in only a few cases were fresh cases of the disease discovered.

ACKNOWLEDGMENTS.

Dr Klein, F.R.S., has given us valuable assistance in the diagnosis of rat-plague, and, before the commencement of the enquiry, Dr C. J. Martin, F.R.S., and Dr Rowland kindly allowed us to make post-mortem examinations of a large number of rats which had died from experimental inoculation with plague bacilli.

The Lister Institute kindly seconded Dr G. H. Macalister, who had already gained extensive experience in the post-mortem examination of rats, to act as one of our assistants throughout the enquiry. He and his colleague, Dr Brooks, though engaged upon work which was extremely unpleasant, tiring, and of long duration, always exhibited remarkable skill in the selection of the material which they submitted to us for diagnosis.

Dr Pringle, Medical Officer of Health for Ipswich, was most helpful in facilitating arrangements at the Ipswich laboratory, the use of which for the purpose of the enquiry must, we fear, have caused him personal inconvenience in the prosecution of his own bacteriological work. Dr Heath, then Medical Officer of Health for East Suffolk, gave us invaluable advice and assistance throughout the enquiry by placing at our disposal his experience of rat-plague in the district which we were investigating. Dr Hollis, Medical Officer of Health for Woodbridge rural district, and Dr Redpath, Medical Officer of Health for Woodbridge urban district, also took great interest in the enquiry and assisted us in the examination of areas likely to prove plague infected.

The Chief Constables of East Suffolk, West Suffolk, Essex, and Colchester gave us every possible facility for the collection of rats through the aid of the police; and the highly efficient organisation of their forces is responsible in large measure for the maintenance of a satisfactory supply of rats throughout the enquiry. The individual constables undertook the extra work which devolved on them with willingness and enthusiasm, and spared neither time nor trouble in obtaining supplies of rats from the farms on their beats. Special thanks are due to Captain Mayne, Chief Constable of East Suffolk, and his

assistant, Superintendent Staunton, for their keen personal interest in the enquiry and their skill in organisation.

Throughout the four months of the enquiry Mr Gordon Merriman worked daily with the rat-catchers for the purpose of collecting and investigating the fleas from the freshly killed animals. His practical advice, based on the local knowledge thus gained and on his personal experience of ratting, added much to the efficiency of our control over the daily work of the rat-catchers.

SUMMARY.

During the period July 1st—October 31st, 1911, 15,332 rats were examined for plague infection. These rats were obtained (*see* Map facing p. 311) partly from the area which in the previous year had been found to be infected with rat-plague and partly from the districts immediately adjacent to this area.

As a result of the present enquiry 27 farms or other premises were found to harbour plague-infected rats.

These 27 places are all within the area previously pronounced to be infected. No plague-infected rats have been discovered outside this area.

Experience of rat collection during the present enquiry showed that the rat destruction which had been maintained by local enterprise since the end of 1910 had in many localities appreciably diminished the rat population.

REPORT UPON THE POST-MORTEM EXAMINATION
OF RATS AT IPSWICH¹.

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INTRODUCTION.

ON June 30th, 1911, we proceeded to Ipswich, with instructions from the Local Government Board to assist in an enquiry, the purpose of which was to determine the extent of the plague epizootic amongst rats in the surrounding districts. The share of this work allotted to us consisted in examination, at the Municipal Laboratory, of the animals received, and in selection, for further investigation by the Board's pathologists in London, of material which appeared suggestive of plague. For this purpose, rats presenting features in any degree consistent with the presence of plague were regarded by us as suspicious.

PRELIMINARIES.

The rats were delivered at the laboratory in boxes. These were opened, and the rats taken out and affixed to dissecting boards. Dissections were made so as thoroughly to expose the principal lymphatic glands, thoracic and abdominal viscera. After inspection, rats obviously free from plague and in other respects devoid of interest, were removed to suitable receptacles, pending transference to the destructor. Those of pathological interest, either with regard to the possibility of plague-infection or from other cause, were set apart for further scrutiny.

For purposes of identification and reference, all rats were numbered.

¹ Reprinted by permission of H.M. Stationery Office.—ED.

Rats set aside on the strength of gross post-mortem appearances as possibly infected with plague were submitted to a complete microscopical examination. Films from heart-blood, liver, spleen and lymphatic glands, stained with carbol-thionin blue, were searched for the presence of organisms resembling plague bacilli. After this, the evidence, macroscopic and microscopic, was reviewed, and the rat was either rejected or reserved for cultural and inoculation tests. In the latter case, the liver, spleen and any other portions indicated by the post-mortem characters were packed and sent to London, accompanied by cards describing the pathological features presented by the animal from which they were taken. Cultivations were usually taken from the organs before despatch. Rats which had been so treated were then immersed in strong formalin, and removed separately in tins for destruction. A record was kept of other features of pathological interest.

NUMBER OF RATS, &C. EXAMINED.

Summary of Results.

Sexes and Pregnancies :

Males	7,490
Females	7,342
Pregnant Females	1,213
Average number of Foetuses	9.9

Species :

<i>Mus decumanus</i>	15,167
<i>Mus rattus</i>	165 ¹
Ferrets	2
Moles	3
Hares	3
Stoat	1
Weasel	1
Rabbit	1

Plague infection.

	Killed	Found dead	Total
Rats examined	15,263	69	15,332
Sent up as suspicious, and proved negative	105	11	116
Proved plague infected by inoculation ...	21	14	35

¹ This number includes 134 rats caught on board ships in the port of Ipswich, and 31 caught in and around the docks.

The work was performed by us with the help of two attendants, and we wish to record our appreciation of the valuable and conscientious service rendered by J. Yerrell, our chief assistant.

POST-MORTEM APPEARANCES.

The accompanying tables show diagrammatically the characters of the selected rats, those found upon further enquiry in London to be infected with plague being placed in Table I, whilst particulars regarding those which proved to be negative are set forth in like manner in Table II. A certain number of rats which were sent up and found to be negative are excluded from Table II for reasons hereinafter stated. (*See* p. 321.) The following paragraphs contain some amplification of these tabular statements.

Plague-Infected Rats.

Lymphatic Glands.—Enlargement of glands was present in most infected rats ; three only failing to exhibit this feature. It was confined to the submaxillary region in five instances, whilst four rats showed congestion of these glands with no increase in size. On eight occasions, the lymphatic glands showed general congestion, but no enlargement. In some of the rats the enlarged glands were associated with minute ecchymoses upon the surface or on section, and definite haemorrhages were observed in a few cases. These appearances were accompanied in some instances by a peripheral zone of congested tissues surrounding the gland, and paling off into a less-marked degree of general subcutaneous congestion (2876 and 3430). In one rat (2876) cross section of an enlarged and congested inguinal gland displayed a necrotic centre. In another (15,079) the left pelvic gland was necrotic. Involution forms, resembling those of *B. pestis*, were observed in some cases, upon microscopic examination of smears from enlarged glands (2876, 11,724, 12,013, and 13,663). Bipolar bacilli were demonstrated in 23 cases.

Subcutaneous Congestion. Congestion, distributed universally over the subcutaneous tissues, was seen in 24 cases. In two instances (2064 and 2522) it was described as being confined to the thoracic and axillary regions. In four cases only was it altogether absent. This feature was very generally present to a marked degree in the infected rats obtained during the concluding period of the investigation. In general, the congestion was manifested as a uniform subcutaneous blush due to distension

of the capillaries, but in a few cases venous engorgement was most prominent, dilation of individual venules being obvious.

Haemorrhages.—Small haemorrhages were found in 10 rats, but in no case was this feature conspicuous. Four instances of retroperitoneal haemorrhage, the pelvic glands being invaded in two cases, were observed. The lungs showed minute ecchymoses on two occasions. Effusions of blood into the bronchial glands and mesentery, and into the subcutaneous tissues of the axillary and submaxillary regions were also described.

Pleural Effusion.—In three plague-infected rats there was no effusion into the pleural cavity. In one putrid rat turbid fluid was found; four cases showed blood-stained effusion. In all the remaining cases a clear pleural effusion, usually considerable in amount, was observed.

Liver.—The pathological changes exhibited by the liver varied greatly in degree and in quality, variations in size, colour and consistency, surface and surface markings being observed. In two rats, both somewhat decomposed, the liver was not described as abnormal. Three rats (2064, 5381, and 13,817) presented livers which appeared at first sight to be normal, but scrutiny with a hand lens showed the presence of fine pitting distributed universally over the surface of the organ, indicative, possibly, of fibrotic change. In a fourth animal (13,663) this pitting was accompanied by signs of congestion. Enlargement, with congestion, was recorded on nine occasions. No register was kept of variations in the degree of consistency of the organ, as this depended to a large extent upon its state of preservation. In 17 cases, the liver is said to be mottled. The term “mottling,” as applied to liver changes, is not intended to indicate any specific pathological conditions, but is used to indicate a mottled or marbled appearance of the organ associated with abnormal definition of the liver lobules. This condition was often shown by livers undergoing putrefactive change (*e.g.* 10,848).

Granular livers, in which were present necrotic foci appearing as scattered whitish points, were found in 18 cases. In two instances the liver is described as granular but no obvious areas of necrosis were observed. It was noticed in one rat (8086), which had been found dead, that although the liver showed advanced putrefactive changes, whitish points of necrosis stood out clearly from the discoloured surface. The manner in which these various features—congestion, mottling, and necrotic change—were combined in different cases, is shown in the table; but it may here be stated that the complex to which has been applied the name of “typical pest liver” (*i.e.* an enlarged, pale, mottled

liver covered with numerous necrotic points) was found only in three cases (2876, 5381, and 12,012).

In every animal except one (1264) microscopical examination showed the presence of bipolar bacilli in liver smears, well-marked involution forms being observed in a number of cases. In two livers (2522 and 11,407) bipolar bacilli were seen in relatively small numbers, together with a large variety of putrefactive forms. In Rat No. 8086 referred to above, very large involution forms of the swollen and globular types were observed.

Spleen.—In eight cases the spleen was macroscopically normal. Fourteen rats showed spleens which presented signs of congestion, being dark in colour and firm in texture. Fifteen exhibited various degrees of granular change. Enlarged spleens showing no distinctive pathological change were not regarded as abnormal. In two cases (2876 and 13,817) coarse punctate necrotic foci were observed. In one rat (1030) the spleen showed a white infarction.

Microscopical examination showed the presence of bipolar bacilli in 26 cases, enormous numbers being observed in seven of these. Well-marked involution forms were found in several instances (*e.g.* 8086). Spleen smears from seven rats did not show any organisms, although with one exception (1264) these were found in corresponding preparations from the liver. It appeared that there was little correlation between the degree of gross morbid change observed and the number of bacilli seen on microscopic examination. In some cases (3430 and 14,855) spleens which appeared normal to naked-eye inspection yielded films found to be crowded with organisms. This condition was met with in the case of the liver in two cases (5382 and 13,817).

Heart Blood.—Films prepared from the heart blood showed no organisms in six cases. The type of organism observed in the remainder was similar to that shown by the smears from liver and spleen. The number varied from very few (in 1264) to enormous quantities (in 1694). The small number which was found in 1264 afforded in that case the only microscopical evidence of plague infection, as the smears from liver and spleen showed no organisms.

Oedema, etc.—Pelvic oedema was seen in one case (1769). Another rat (10,848) showed the rare condition of general subcutaneous oedema. In Rat 13,817 the lungs were markedly engorged, and were found upon microscopical examination to contain large numbers of bipolar bacilli.

Negative Rats.

Particulars are shown in Table II of 76 rats selected as possibly plague infected, which, however, were shown by cultural and inoculation experiments to be negative. Some of the salient features of these rats are summarised below. In addition to the negative rats included in Table II 35 rats were sent up for further investigation: 19 of these exhibited abundant pleural effusion containing numerous bipolar bacilli, and are more fully described below; the remaining 16 were found to harbour bipolar bacilli in their accessory sexual glands. In most rats, even those apparently healthy, smears from accessory sexual glands showed large numbers of organisms, which often exhibited bipolar staining and bore a close resemblance to the plague bacillus. Cultural investigation and inoculations carried out in these cases proved the absence of any such infection. As soon as it was ascertained that neither of the conditions was in any way associated with infection suggestive of plague, such animals were regarded as negative.

Lymphatic Glands.—Glandular enlargement was a very common feature, and was particularly well marked in two cases. Bipolar bacilli were found in gland smears from 21 rats, and in some cases organisms resembling plague involution forms were observed. Enlargement of lymphatic glands, particularly those in the submaxillary region, was observed in a great majority of the rats received for examination, but in most cases, when microscopic examination was made, no organisms were found. The pelvic glands also were as a rule conspicuous in the rats examined during the course of this enquiry, although it had previously been observed by one of us who had the opportunity of examining a series of rats in January, that these glands were not usually obvious at that season. In the majority of cases enlargement of the glands was not accompanied by congestion.

Subcutaneous congestion.—Intense and general subcutaneous congestion was only observed twice. Localised congestion was seen in a number of cases. In 22 rats no signs of congestion were observed.

Haemorrhages.—Haemorrhages were described as occurring in 11 cases. The majority of these were strictly localised and were probably traumatic in origin.

Pleural Effusion.—Clear pleural effusion was observed in 39 cases, the amount of fluid being scanty in the majority of these. Effusion described as blood-stained occurred in 39 instances. In several decomposed rats turbid fluid was found in the pleural cavity. These numbers

include 19 rats not included in Table II. In 12 of these the pleural effusion was blood-stained, and in seven instances it was clear or turbid. Microscopical examination of films prepared from such pleural effusions showed the presence of a great variety of organisms, some of which simulated very closely both typical and pleomorphic forms of plague bacilli. Further, the groupings of organisms on some films bore a most striking resemblance to those characteristic of *B. pestis*. These organisms were subsequently regarded as being putrefactive.

Liver.—The livers showed punctate foci of necrosis in 16 cases, in five of which the feature was well marked. In four such rats infection with the Gaertner bacillus was subsequently demonstrated. Microscopically no organisms were found in 26 cases; 50 showed the presence of bipolar bacilli.

Spleen.—Granular changes in the spleen were frequently observed. In three animals the spleen showed necrotic foci. Bipolar bacilli were found in 42 rats.

Heart Blood.—Films prepared from the heart blood showed cocco-bacilli or bipolar bacilli in 36 rats.

Minor changes in the viscera were observed in a great number of rats which were rejected at once, as well as in those reserved for microscopic examination. Mottling or marbling of the liver due to commencing putrefaction was very common. Minor granular changes in the spleen, overgrowth of submaxillary glands, and effusions of blood into the pleural cavity were noticed with great frequency. No statistical record of such changes was kept, but, in about 600 such rats, microscopic examinations were made. These gave negative results.

OTHER PATHOLOGICAL CONDITIONS.

Gaertner Infection.

In 14 of the cases, where infection with plague was, upon preliminary examination, considered possible, further investigation demonstrated the presence of bacilli of the Gaertner group. The allocation of these organisms was based upon cultural and fermentation reactions.

Five other rats were noticed, which demonstrated so clearly the post-mortem character associated with Gaertner infection, that they were not included among the plague-suspects. Nineteen rats altogether were, therefore, regarded as being infected with this organism. It is probable, however, as the investigation was not directed definitely to

the detection of such cases, that this number does not represent the degree of prevalence of this disease among the rats received.

The most prominent post-mortem features typically associated with Gaertner infection were displayed by the liver and spleen. The former was not as a rule granular, but thickly peppered with white points. It should be noted that although a typical plague-infected liver differs markedly from the appearances presented by the organ in a typical Gaertner infection, in certain cases the post-mortem appearances are of no great assistance in differential diagnosis, and further investigation is needed to determine the nature of the disease under consideration. The spleen, on the other hand, was much more markedly granular than that found in the majority of plague-infected rats. Microscopically the number of organisms seen was usually small, but these exhibited, not infrequently, bipolar staining. Some slight amount of subcutaneous congestion was shown by a number of Gaertner-infected rats.

Trypanosomiasis.

Trypanosomiasis was very common, particularly during the first two months of the investigation. The most general and prominent indication of this infection was blood-stained pleural effusion consisting in many cases of pure blood. Rats exhibiting such a blood-stained effusion, whilst appearing fairly healthy in other respects, might with some degree of certainty be regarded as infected with trypanosomiasis. A continuous series of 2000 rats received in August were investigated particularly with regard to this point. Of these, 145 showed pleural effusion which was decomposed and putrid in many cases, and clear in some others, while in 55 cases the fluid was blood-stained: 45 of these showed the presence of trypanosomes. These parasites were also found in five cases where the pleural effusion was turbid. Incidentally, three rats in this series of 2000 were found to be plague-infected. In these three (2522, 2876, 3346) the effusions were severally turbid, clear, and blood-stained. Other appearances, associated with trypanosomiasis, were congestion of the subcutaneous tissues and lymphatic glands, enlargement of glands, and congestion of the liver. In one case these features were particularly obvious, and the right inguinal gland, particularly, was enlarged and surrounded by a zone of deeply congested tissues. Microscopic examination of this gland showed the presence of many trypanosomes, the agglutination phenomenon being well demonstrated. Large numbers of trypanosomes were found also in the heart

blood. This rat was interesting, as showing that the infection was introduced probably within the area drained by the right inguinal gland. It was also very important, in that the post-mortem appearances presented a picture very closely resembling that associated with plague. Agglutination of trypanosomes was, on another occasion, found in a film prepared from the turbid pleural effusion.

Other Infections.

Suppurative conditions of the lungs occurred with great frequency. This phenomenon is extremely common amongst rats, and has been observed on every occasion when large numbers have been examined. In one series, all stages of the condition were seen, from the earliest, presenting a few small foci containing glairy material, to advanced stages in which the lung tissue is almost entirely replaced by caseous matter, the healthy lung which remains being thereby compressed and displaced and the bony wall of the thorax deformed. Suppurations, general and localised, in the abdominal cavity were not infrequent, and abscesses in lymphatic glands were very common. Osteomyelitis of the hind-limb bones was twice found.

Twenty-five cases of rat-leprosy were observed.

DISCUSSION.

Some facts, observed in the study of both negative and positive cases, may illustrate certain difficulties of preliminary diagnosis, which assume their greatest importance in the investigation of outbreaks of rat-plague occurring in this country.

Infected rats presenting atypical appearances were relatively common, particularly during the early part of the enquiry, at the commencement, that is, of the seasonal epizootic. On the other hand, those found during the last two months of the investigation showed features typical of plague. Broadly speaking, preliminary diagnosis during the first period of two months was usually tentative, while during the second period it was, in the great majority of instances, certain. It must not be assumed, however, that this statement implies that negative cases could always be diagnosed as such without resort to animal experiment. Even in regard to cases in which the diagnosis appeared certain, a caveat must be entered. A study of the characters

TABLE I. *Positive Cases.*

Under the heading Macroscopic Appearances the plus and minus signs indicate degrees of enlargement, or other abnormality, thus:

- = normal.
 ± = slight.
 + = definite.
 ... = not examined.

Under the heading of Microscopical Appearances, these signs refer to plague-like bacilli, thus:

- = nil.
 ± = few.
 + = some.
 ... = very many.
 +++ = not examined.
 D = found dead.

An asterisk in the column relating to pleural effusions, signifies "blood-stained."

K = killed.

Serial No.	Macroscopic appearances										Killed or found dead					
	Glands			Liver			Spleen		Microscopic appearances							
	Enlargement	Congestion	Subcutaneous congestion	Hemorrhages	Pleural effusion	Necrosis	Mottling	Congestion	Necrosis	Granulation		Congestion	Glands	Liver	Spleen	Heart blood
1030	+	-	-	-	-	+	+	-	-	-	-	...	+	-	+	K
1261	-	-	+	-	*	+	+	-	-	+	-	...	+	-	±	K
1691	+	±	+	+	*	+	±	-	-	+	-	...	+	±	+	D
1694	+	+	+	-	±	+	-	+	-	-	±	-	+	+	+	D
1769	+	+	+	+	±	-	-	-	-	-	-	...	+	+	+	D
2064	+	+	+	+	±	-	-	-	-	-	-	...	+	-	+	K
2156	±	-	+	+	+	-	-	-	-	+	-	...	+	-	-	D
2522	+	-	±	+	+	-	+	-	+	+	+	-	±	-	±	D
2876	+	-	+	+	*	+	+	-	+	-	+	+	+	+	...	D
3346	+	+	-	+	-	-	+	-	-	-	-	+	+	+	+	D
3430	+	+	+	+	+	-	+	+	-	-	+	+	+	+	+	D
4849	+	+	+	+	+	-	+	+	-	-	+	+	+	+	+	K
5381	+	-	±	+	±	+	+	+	-	±	+	+	+	+	+	K
5382	+	+	+	+	-	+	-	-	-	+	-	±	+	±	-	K
7643	+	+	+	-	+	-	-	-	-	+	-	±	+	+	+	D
8086	-	-	+	+	+	+	-	-	-	+	-	...	+	+	+	K
10169	+	+	+	+	±	+	+	±	-	+	+	...	+	+	+	K
10840	+	+	+	+	±	+	+	-	-	+	+	...	+	+	+	D
10848	+	+	+	-	+	+	-	±	-	+	+	...	+	+	+	K
11407	-	-	-	-	+	-	±	-	-	-	-	...	+	+	+	K
11724	+	+	+	-	±	-	+	-	-	-	-	...	+	-	+	K
12012	+	+	+	+	+	+	+	+	+	+	+	...	+	+	+	D
12013	+	+	+	+	+	+	+	-	-	+	+	...	+	+	+	K
12245	+	+	+	+	+	+	+	+	+	+	+	...	+	+	+	K
13663	+	-	+	+	±	-	+	-	-	±	+	...	+	+	+	D
13712	+	+	+	-	+	+	-	±	-	±	+	+	±	+	...	D
13713	+	+	+	-	+	+	+	-	-	±	+	+	+	+	+	K
13817	+	+	+	-	+	+	+	-	-	±	+	+	+	+	+	K
14199	+	+	+	+	+	+	-	-	-	±	+	+	+	+	+	K
14816	+	-	±	+	+	±	-	-	-	±	-	+	+	+	±	K
14854	+	-	+	+	+	+	-	+	-	±	+	+	+	+	+	K
14855	+	-	+	+	+	+	-	+	-	±	+	+	+	+	+	K
14911	+	+	+	+	+	+	-	+	-	±	+	+	+	+	+	D
15079	+	+	+	+	+	+	+	+	-	±	+	+	+	+	+	K
15219	+	-	+	+	+	+	-	+	-	±	+	+	+	+	+	K

TABLE II. *Negative Cases.*

Serial No.	Macroscopic appearances										Killed or found dead					
	Glands			Liver		Spleen		Microscopic appearances								
	Enlargement	Congestion	Subcutaneous congestion	Haemorrhages	Pleural effusion	Necrosis	Mottling	Congestion	Necrosis	Granulation		Congestion	Glands	Liver	Spleen	Heart blood
51	+	-	±	+	+	-	+	+	-	-	±	+	-	-	+	K
92	+	-	-	+	-	+	+	-	-	±	±	-	-	-	+	K
102	+	-	-	-	-	-	+	-	-	-	+	-	-	-	-	K
334	+	-	-	-	+	-	+	-	-	-	+	-	-	-	-	K
444	+	-	-	-	+	-	+	-	-	-	+	-	-	-	-	K
544	+	-	-	-	+	-	-	-	-	-	+	±	-	-	+	K
550	+	-	+	+	+	+	-	-	-	+	+	-	+	-	-	K
669	-	-	±	+	+	+	+	+	-	-	+	-	+	+	-	K
711	+	+	±	+	+	+	±	-	-	-	-	±	+	+	-	K
723	+	+	±	+	+	-	±	-	±	-	-	±	+	+	-	K
752	+	+	±	+	+	-	±	-	-	-	-	±	+	+	-	K
818	+	+	±	+	+	-	±	-	-	-	+	±	-	-	-	K
920	+	+	±	+	+	-	±	-	-	-	+	±	-	-	-	K
1444	+	+	+	-	±	±	-	-	-	-	-	-	±	-	-	D
2557	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	D
2651	±	+	+	-	+	-	-	-	-	-	-	+	-	+	+	K
2952	+	+	+	±	±	-	-	+	-	-	-	-	-	-	-	K
2994	-	+	-	-	+	-	+	+	-	-	-	-	-	+	+	K
3023	+	-	+	-	+	-	+	-	-	-	-	-	-	+	+	K
3030	+	-	+	-	+	-	+	+	-	-	-	-	-	+	+	K
3031	+	-	-	+	+	+	+	+	-	-	-	-	+	+	+	K
3051	-	-	-	+	+	-	±	-	-	-	-	-	+	+	+	K
3052	-	-	-	+	+	-	±	-	-	-	-	-	+	+	+	K
3053	-	-	-	+	+	-	±	-	-	-	-	-	+	+	+	K
3056	+	-	+	+	+	-	±	-	-	-	-	-	+	+	+	K
3281	+	+	-	+	+	-	±	-	-	-	-	-	+	+	+	K
4187	+	+	+	+	±	-	±	-	-	-	-	+	-	-	-	K
4242	+	+	+	+	+	+	+	-	+	+	±	+	-	±	+	K
4374	-	+	+	-	-	+	+	-	-	-	-	+	-	-	±	D
4549	+	+	±	-	+	-	-	-	-	-	-	+	±	-	+	K
4659	+	+	+	-	-	-	-	-	-	-	-	+	+	+	+	K
4667	+	+	+	-	+	-	-	-	-	-	-	-	+	-	±	D
4761	+	-	+	+	±	±	+	+	-	-	-	-	+	+	+	K
4843	+	+	+	+	+	±	±	+	-	+	+	+	+	+	+	K

shown by some rats (4843, 4886, and 10,150), in Table II, will illustrate the danger of making positive diagnoses upon the evidence afforded by post-mortem features, not confirmed by inoculation experiments. Reference, on the other hand, to three cases (1264, 2064, and 11,407), in Table I, will illustrate the necessity of care in the investigation of epizootics where rats presenting such slender evidence of infection may occur. Reports of similar investigations carried out at Hamburg and elsewhere indicate that such atypical plague rats may always be encountered in the investigation of European epizootics.

The individual features of greatest service for diagnosis were subcutaneous congestion, clear pleural effusion, and granular, necrotic changes in the liver. All three were present, usually to a marked degree, in most of the plague-infected rats, but only in 9 rats found to be negative. Pleural effusion, either clear or blood-stained, and congestion were observed in a considerable number of cases. No rat found positive failed to display obviously one or more of these characters. In order of relative value, subcutaneous congestion and pleural effusion would come first, the liver changes being less important, but their collective significance should be considered rather than the individual prominence of each single factor.

Enlargement of lymphatic glands possesses, generally speaking, little weight as primary evidence. At the season in which the enquiry was carried out, pelvic glands were conspicuous in most rats. Sub-maxillary glands were enlarged as a rule. Congestion in regions where this feature was generally not obvious was of some value as a secondary piece of evidence.

In general the facts observed agree with those which emerge from the great bulk of work which has been published upon the question of plague epizootics.

Reference has already been made to the misleading character of gross morbid changes associated in one case with trypanosomiasis. Enlarged and congested glands, subcutaneous congestion, pleural effusion, engorgement of liver and spleen, all features suggestive of plague, were shown, though microscopic examination demonstrated readily the real nature of the infection.

In one case a dual infection of pest and trypanosomiasis was present, and it is difficult to say how far each infection was responsible for the post-mortem appearances observed.

The peppery liver associated with Gaertner infections is a crisply defined pathological entity, but in many cases this organ presented

appearances which caused trouble in diagnosis. Concurrent microscopic and macroscopic evidence gave in most cases clear indication of the true nature of the infection, but atypical livers and spleens showing signs of congestion with minor necrotic and granular changes were in a number of instances reserved for inoculation purposes. The result of inoculations with such material showed the importance of microscopic examination, for negative results were obtained in every case where no bipolar bacilli were found in the films.

Microscopical examination presents, however, a number of fallacies. Bipolar staining is at times shown in intestinal and putrefactive organisms, as well as by certain bacteria, probably of intestinal origin, which are found in the accessory sexual glands of otherwise healthy rats. Putrefactive organisms may also present forms resembling very closely pleomorphic plague bacilli, and assume groupings usually regarded as characteristic of *pest.* These were best seen in decomposed pleural effusions. Inoculation of such material from 16 cases—not recorded in Table II—showed the fallacious nature of such appearances. As previously mentioned, many rats not infected with plague revealed organisms in the liver and spleen which could not be differentiated from plague bacilli on microscopical examination alone.

In the infected rats generally, plague bacilli were less numerous in blood films than in spleen smears, but in a few cases the reverse held true. In some rats received in October plague bacilli were present in enormous numbers in the blood. In one or two of these, the experiment was tried of cutting off an inch of tail and preparing a film from the cut surface of the stump. Films crowded with plague bacilli were thus obtained. This point has a practical application, indicating that the practice, prevalent amongst those who catch rats, of cutting off the tails is not without danger. The cut stump presents a sharp vertebral point which might readily inflict an inoculation scratch.

CONCLUSIONS.

1. Many plague-infected rats in the earlier period (July and August) of the epizootic presented atypical appearances.
2. The majority of those found in the later period were typical and could usually be diagnosed upon post-mortem examination with a considerable degree of certainty. A number of the negative cases presented difficulty in diagnosis throughout the whole enquiry.

3. Features suggestive of plague infection may be simulated by rats infected with trypanosomiasis or with bacilli of the Gaertner group.
4. The most valuable diagnostic points are the presence of subcutaneous congestion, pleural effusion, and necrotic changes in the liver. These should be considered collectively.
5. Minor granular and necrotic changes in liver and spleen uncorroborated by other suggestive pathological characters, macroscopic and microscopic, are in general not indicative of plague.
6. Major granular and necrotic changes in liver and spleen are very suggestive, but not necessarily indicative, of pest infection.
7. Fallacies in direct microscopic diagnosis are presented by certain intestinal and putrefactive organisms.

THE INFLUENCE OF METABOLIC FACTORS
IN BERI-BERI. PART I. THE EFFECT OF
INCREASING THE CARBOHYDRATE RATION
ON THE DEVELOPMENT OF POLYNEURITIS
IN BIRDS FED ON POLISHED RICE.

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It is now taken as definitely settled that the disease beri-beri is essentially privatory in origin, resulting from the deficiency in the food supply of a substance or substances the precise nature of which is at present unknown. If this simple explanation were complete it would follow that the more rapid and thorough the withdrawal of the active substance, the speedier would be the onset of the disease and the more severe its effects.

Maurer (1907) found, however, that when fowls were fed on polished rice those birds receiving the largest quantity of rice were the first to develop symptoms of polyneuritis and that by feeding small amounts of rice the disease could be postponed for a long time.

Chamberlain, Bloombergh, and Kilbourne (1911) on the other hand found that birds consuming large rations of polished rice remained free from polyneuritis for a longer time than birds eating little or no rice at all.

Cooper (1913) however obtained results similar to Maurer, finding that pigeons eating very small amounts of polished rice developed symptoms of polyneuritis in from 20 to 33 days, while those birds receiving daily rations of rice exceeding $1/30$ th their initial body-weight fell ill in from 9 to 26 days. No constant difference however was noticed in the periods elapsing before the appearance of polyneuritis in the

case of the pigeons receiving daily rations of polished rice varying from 1/11th to 1/30th the initial body-weight.

Caspari and Moszkowski (1913) made observations of a similar nature. They found that pigeons fed on hens' eggs, grape-sugar, and salt remained quite healthy and increased in weight, but pigeons fed on the same rations of egg, sugar, and salt with the addition daily of polished rice developed polyneuritis.

Funk (1914) has still more recently obtained similar results to Maurer (1907) and Cooper (1913), having found that birds fed on large rations of polished rice develop polyneuritis in a shorter time than birds fed on small rations. He also fed series of pigeons on dietaries containing respectively excess of starch, sugar, protein, and fat, and found that the birds receiving the large rations of starch were the first, while those receiving excess of fat were the last, to develop polyneuritis, the birds fed on large rations of sugar and protein falling ill intermediately.

It is thus seen that when the food ration is increased by the addition of material, *e.g.* polished rice, with a very low content of anti-neuritic substance, although the daily allowance of active substance is slightly increased, the development of polyneuritis is actually accelerated.

Similarly birds deprived of everything but water and therefore receiving no anti-neuritic substance at all may live longer than birds fed on a diet, *e.g.* polished rice with a very low content of the active substance, and may die finally without any indication of polyneuritis, although occasional cases of polyneuritis with fatty nervous degeneration were observed by Chamberlain, Bloombergh, and Kilbourne (1911) in fowls. The animal supplied with a little of the substance thus succumbs to the disease, but that receiving none at all may fail to do so. It has been suggested that the starved animal drawing upon its own tissues for a supply of combustible material obtains an adequate amount of anti-neuritic substance in the process of tissue destruction. This is a plausible explanation of the phenomenon, but it does not account for the results of Experiments VI, X and XI (in the present paper) which show first that birds fed on rations equal to 1/20th of their body-weight of washed polished rice lost as much as 40 per cent. in weight and yet developed polyneuritis, and, secondly, that a supply of anti-neuritic substance adequate for the organism provided that the carbohydrate ration was maintained at a certain magnitude was unable to avert polyneuritis when the carbohydrate ration was increased.

Certain facts concerning human beri-beri are parallel to these experimental observations made in the bird. Thus paradoxically, it has been

found by nearly all observers (*Cause and Prevention of Beri-Beri*, Braddon, 1907, p. 256) that in epidemics of beri-beri the well nourished are the first to succumb and are actually more liable to the disease than the underfed. Similarly, under natural conditions men, owing to their larger energy output, partake of polished rice much more freely than women and are more liable to beri-beri. In institutions however where men and women have the same fixed rations the sexes are equally susceptible to the disease. Again, in prisons and asylums in which the dietary has been varied from time to time, every increase in the polished rice component of the diet, the rations of other foodstuffs remaining the same, was attended by increased beri-beri. From these observations Braddon drew the conclusion that in rice-eating communities the extent and severity of beri-beri vary directly with the quantity, absolute or relative, of polished rice consumed.

Admitting, then, that in the production of beri-beri deprivation of a certain substance is an essential factor, it is of obvious importance to ascertain the precise rôle which the active substance plays in metabolism, and to determine, if possible, the quantitative relations which the intake of it must bear to the other components of the dietary, *i.e.* to obtain some information as to the composition of diets best adapted for promoting, first, a good absorption, and, secondly, an economical utilization of the active substance and yet adequate for maintaining the organism in nutritive equilibrium.

With this end in view and also on account of its special importance in the prevention of beri-beri the effect upon the development of polyneuritis in birds of increasing the carbohydrate ration of a dietary containing a certain amount of the anti-neuritic substance has been studied quantitatively and the results obtained are set forth in this communication.

Experiments I to XII were carried out by one of us (W. L. B.) at Seremban, Federated Malay States, during the years 1909-11 and the results are borne out by the remaining experiments which were undertaken by the other of us (E. A. C.) at the Lister Institute during 1913.

I. *The effect of rice-diets upon the health and body-weight of birds.*

Experiment I. Padi as an exclusive food for fowls.

Padi, the whole unhusked rice grain, containing at the best about seven per cent. of protein and one per cent. of fat, is by no means a good food for birds, but it was found that fowls fed daily on 1/20th their body-weight of padi could be maintained in fair health for from 60 to 100 days,

after which they usually died without however any indication of polyneuritis, although some had lost considerably in weight.

TABLE I.

Bird	Change in weight by 30th day
1	- 20 per cent.
2	+ 18 "
3	- 37 "
4	+ 10 "
5	- 23 "
6	- 23 "
7	- 24 "
8	+ 27 "
9	- 7 "
10	- 7 "

Experiment II. Cured or unpolished rice.

Cured or parboiled unpolished rice contains all the elements of the original padi except the husk and coloured layer of the pericarp, which are rubbed off together after the process of boiling or steaming and subsequent drying. This rice is about 10 per cent. richer in protein and fat than padi.

Four fowls fed on 1/20th their body-weight of this rice remained free from polyneuritis, but died on the 38th to 78th day of the experiment of intercurrent disorders.

Six birds fed on rations of "cured" rice varying from 1/10th to 1/26th initial body-weight with two exceptions maintained their weight for four weeks as indicated below.

TABLE II.

Bird	Ration	Change in weight by 26th day
85	1/26th body-weight	0
86	1/13th "	- 17 per cent.
87	1/10th "	0
88	1/16th "	0
89	1/15th "	- 17 per cent.
90	1/12th "	0

Experiment III. "Fresh" rice.

Fresh rice, or partly polished hand-pounded rice prepared by the Malay natives for home use, retains portions of the inner pericarpal layer or silver skin, as well as much of the aleurone layer which contains about half the total proteids and fats of the seeds. Hence the rice

has a food value almost as good as that of padi or parboiled rice. Fowls fed naturally on this rice, *i.e.* on rations less than 1/20th body-weight, maintained body-weight and remained healthy for at least one month, as shown in the following table.

TABLE III.

Bird	Change in weight by 30th day
55	0
56	+ 35 per cent.
57	0
58	0
59	- 40 per cent. (Died, but no polyneuritis)
60	0

The rice, such as was used in the above experiments, in which the surface layer of the endosperm is wholly preserved, contains on an average 7 per cent. of protein and 80 per cent. of carbohydrate. Roughly it may be said therefore that physiological balance could be maintained on a diet of which protein composed about 1/300th and carbohydrate 1/25th the body-weight of the birds when sufficient anti-neuritic substance was provided.

The following experiments illustrate what happens, first, when the supply of anti-neuritic substance is reduced, the other food materials remaining constant, and, secondly, when the intake of active substance is kept constant and the carbohydrate ration increased.

Experiment IV. Polished Siam Rice, 1/20th body-weight.

Fowls fed on the above ration developed polyneuritis in from 24 to 69 days, six birds falling ill on the 24th, 25th, 42nd, 43rd, 47th, and 69th days respectively.

It is seen that this rice induced polyneuritis somewhat slowly, but a comparison of the results with those obtained in Experiments I to III indicates that its content of anti-neuritic substance was much smaller than that of padi, cured, or fresh rice.

Experiment V. Washed polished Siam rice, 1/20th body-weight.

In this experiment the rice was first soaked in excess of water for 24 hours. Fowls fed on the above rations developed polyneuritis in from 10 to 35 days, six birds falling ill on the 10th, 13th, 14th, 24th, 32nd, and 35th days respectively. It is seen that this rice induced

polyneuritis more rapidly than the original polished rice, indicating that it had been to a considerable degree deprived of active material by the process of soaking.

Experiment VI. Washed polished Siam rice, 1/20th body-weight.

In this experiment the rice was soaked in excess of water for 48 hours before feeding, and fowls fed on the usual ration developed polyneuritis in 15 to 17 days (15, 15, 15, 15, 16, 17). By the prolonged washing the content of anti-neuritic substance was thus still further diminished if not wholly cut off, and polyneuritis was greatly accelerated. The loss in weight in the case of these birds amounted to as much as 25 to 40 per cent. at the end of the fortnight and in general exceeded anything observed in the other series as shown in the following table. This indicates apparently that the changes culminating in the final break-down (polyneuritis) begin immediately with the deprivation of the protective substance.

TABLE IV.

Bird	Change in weight at end of 14 days			
	Padi 1/20th body- weight ration	Cured rice 1/10th—1/26th	Fresh rice (less 1/20th body-weight) (partly-polished)	Siam pol. rice washed 48 hrs. 1/20th body-weight
1	- 30 %	+ 4 %	0	- 40 %
2	0	- 4	+ 20 %	- 36
3	- 35	- 5	+ 5	- 23
4	0	- 6	0	- 25
5	- 23	- 14	- 20	- 40
6	- 30	- 5	+ 3	- 35
7	- 26			
8	- 10			
9	- 7			
10	- 10			

The following experiment corroborates these results and also illustrates a point of great practical importance.

Experiment VII.

Ten fowls were fed on 1/20th their body-weight of cured or parboiled rice which had been previously soaked in cold water for 24 hours. Nine of the birds developed polyneuritis in from 13 to 39 days as will be shown in Table V, although birds fed on the same ration of the original parboiled rice had not developed polyneuritis on the 38th to 78th days.

The custom of soaking rice before cooking and then discarding the water has been practised in some institutions and has actually been accountable for severe epidemics of beri-beri amongst the inmates.

In the case of two of the 10 fowls fed on the soaked parboiled rice post-mortem examination revealed general oedema, hydropericardium, and distension of gall bladder, appearances rarely occurring in avian beri-beri. This suggests that further experiments with this rice may lead to the differentiation of the factor which determines the occurrence of oedema in human beri-beri.

The effects of the various rices upon the health of birds are classified in the following table.

TABLE V.

Rations 1/20th body-weight.

No.	Padi	Cured Rice (unpolished rice)	Fresh Rice (partly polished by hand)	Polished Siam rice			Cured rice Soaked for 24 hrs.
				1 Original	2 Soaked in excess of water 24 hrs. Polyneuritis appeared on	3 Soaked for 48 hrs.	
1	Died on 67th day. No polyneuritis	Died on 38th day. No polyneuritis	All healthy after 4 weeks	24th day	10th day	15th day	13th day
2	Died on 72nd day. No polyneuritis	Died on 47th day. No polyneuritis	...	25th ..	13th ..	15th ..	15th ..
3	Died on 93rd day. No polyneuritis	Died on 54th day. No polyneuritis	...	47th ..	14th ..	16th ..	18th ..
4	Died on 96th day. No polyneuritis	Died on 78th day. No polyneuritis	...	42nd ..	24th ..	15th ..	26th ..
5	Still healthy on 100th day	43rd ..	32nd ..	16th ..	32nd ..
6	69th ..	35th ..	17th ..	39th ..
7	16th ..
8	23rd ..
9	25th ..
10	26th ..
11	34th ..
12	Died on 63rd day without polyneuritis

Experiments IV to VII prove no more than is well known that the consumption of rice deprived of a certain substance induces beri-beri. The observations however have value according to the light in which they are regarded. In Experiments I to III the birds were fed on a ration containing a definite amount of starch and a definite amount of

anti-neuritic substance, the association of which with the starch prevented polyneuritis. In Experiments IV to VII it is seen that the ration of carbohydrate remaining constant, while the supply of active substance was reduced or withdrawn, beri-beri followed and the effect varied directly with the extent of withdrawal.

The first three experiments furnish a rough index of the normal quantity of anti-neuritic substance necessary daily to maintain physiological balance in a fowl when a normal ration of food is ingested. This quantity is the amount contained in a bulk of padi equal to 1/20th the body-weight of the bird.

Experiments were next made with commercial starch such as is sold for laundry purposes. 10 gms. daily were given alone to fowls of 300 to 500 gms. and the results are tabulated below.

*Experiment VIII. Five fowls fed on a ration of 10 gms.
of pure starch only, daily.*

TABLE VI.

No.	Day, and weight in grammes											Results
	1	4	6	8	10	12	14	16	18	20	24	
37	525	510	570	600	540	480	433	450	360	375	345	Became weak on 16th day and remained so till 20th but no signs of beri-beri. Food changed to padi but died 24th day.
49	375	375	405	360	240	—	—	—	—	—	—	Sickened but no signs of paralysis, died 10th day.
50	300	270	365	300	300	240	225	173	—	—	—	Gradually wasted, on 14th day very weak but no paralysis. Died 16th day.
51	480	435	465	450	390	390	463	345	—	—	—	Wasted and weak but able to run, transferred on 16th day, recovered.
52	465	450	465	413	435	390	360	360	345	300	—	On 20th day very thin and weak but no paralysis; somewhat somnolent. Food changed. Recovered.

Although considerable loss of weight took place in each of these birds, none of the ordinary symptoms of avian beri-beri were noted up to the time when they became critically ill.

The condition seemed to be (after the first few days) one of simple starvation. It appears probable that, although at first some of the starch given was digested, as the increases of weight in some of the birds indicates, later on absorption ceased, possibly for mechanical reasons—the starch being apt to make a solid mass in the crop and duodenum. As will be seen later exclusive diets of glucose induced polyneuritis in birds and the addition of sago to a diet consisting of polished rice and yeast accelerated the development of the disease, Expt. XV (p. 345).

Experiment IX. Six fowls were fed on a daily ration of 30 gms. starch plus 1 gm. of egg-yolk.

TABLE VII.

No.	Day, and weight in grammes							Remarks
	1	9	11	13	15	17	19	
63	570	480	540	480	420	450	330	Died on 19th day. No paralysis.
65	570	540	540	480	420	—	—	Died on 15th day. No paralysis.
100	660	660	630	540	540	—	—	Died on 14th day. No paralysis.
94	1200	1050	1050	1020	1020	1020	900	Sick on 19th day, legs paralysed.
1	1370	1320	1290	1260	1200	1040	990	Died on 19th day. No paralysis.
10	1500	1340	1340	1380	1350	1350	1260	Quite well and strong on 19th day.

The egg-yolk in this experiment was mixed thoroughly into a paste with the starch and a little water, the mixture being given in pills. Although there were four deaths, in none of the four were characteristic signs of beri-beri observed previously.

One bird, however, developed beri-beri in the subacute form and one remained except for loss of weight quite well at the end of the experiment.

Cooper and Funk (1911) obtained somewhat similar results, pigeons fed on 1/10th to 1/40th daily rations of Coleman's starch, potato-starch, inulin, dextrin, and cane sugar in some cases developed typical symptoms of polyneuritis with considerable fatty degeneration in sciatic nerves and spinal cords. Many of the birds however only exhibited signs of weakness and then died, but even in these there was evidence of fatty degeneration. In all cases the deficiency in the diet of the anti-neuritic substance had evidently made itself felt, but in some cases at the time of death the depletion of the body store of active substance had only proceeded far enough to cause some degree of fatty degeneration, and was not sufficient to induce the development of the typical neuritic symptoms. The proportion of undoubted cases of polyneuritis is given below.

TABLE VIII.

Diet	No. of cases	Distinct cases of polyneuritis	Cases of weakness only
Coleman's starch	3	1	2
Potato starch (pure)	6	1	5
Inulin	3	2	1
Cane sugar	7	1	6
Dextrin	8	5	3

The frequent failure of such carbohydrate diets to induce typical polyneuritis is surprising in view of the facts that the symptoms regularly develop in birds fed liberally on polished rice and even when polished rice or sago is added to a basal ration containing a minimum of the anti-neuritic substance (Experiments X to XV). In the case of starch, as already pointed out, the condition would appear to be one of simple starvation. The case of cane-sugar however offers more difficulty, as it would seem unlikely that saccharose escaped inversion in the alimentary canal. The excreta were however not examined for the presence of saccharose.

The results with dextrin show that exclusive diets of this carbohydrate frequently induced distinct symptoms of polyneuritis. This may be due to the fact that dextrin dissolves in water at ordinary temperatures and would be readily hydrolysed to glucose in the alimentary canal, so that the birds were thus called upon to metabolize large amounts of carbohydrate and depletion of their store of anti-neuritic substance rapidly took place.

Experiments with glucose have since been carried out, and it has been found that of four birds fed exclusively on aqueous solutions of this carbohydrate three developed typical symptoms of polyneuritis, while the fourth only exhibited symptoms of weakness with however considerable fatty degeneration in the sciatic nerves.

II. *The effect of increasing the carbohydrate ration upon the time elapsing before onset of polyneuritis in birds.*

The succeeding experiments show what happens when the intake of anti-neuritic substance is maintained constant, but the proportion of food-material is increased.

Experiment X.

(a) Six fowls weighing about 600 gms. each were fed on a ration of 30 gms. of padi daily ($1/20$ th body-weight) for 45 days.

(b) Another six fowls were fed on the same ration of padi as the above plus 90 gms. of water-extracted unpolished rice ($1/6$ th to $1/7$ th body-weight).

The birds of series (a) maintained their body-weight and were quite healthy at the end of 45 days, but those included in series (b) all developed polyneuritis in from 10 to 16 days.

Experiment XI.

(a) Ten fowls weighing about 600 gms. each were fed on $22\frac{1}{2}$ gms. padi daily (about 1/25th body-weight).

(b) Ten other fowls received the same ration of padi and in addition 54 gms. of polished Siam rice daily (1/10th body-weight approximately).

Nine of the birds fed on padi only were strong and healthy at the end of 30 days, three of them gaining in weight and six losing in weight. The 10th bird escaped early in the experiment.

TABLE IX.

(a) Padi only, $22\frac{1}{2}$ gms. daily.

No.	Day, and weight in grammes							Effect of diet and % change by 30th day
	1	5	8	10	21	27	30	
1	600	510	472½	450	372½	—	480	- 20 %
2	600	630	570	510	630	—	705	+ 18 %
3	600	480	450	360	420	—	375	- 37 %
4	420	500	465	375	450	—	465	+ 10 %
5	570	600	540	420	450	—	442½	- 23 %
6	570	480	472½	390	420	—	442½	- 23 %
7	540	450	420	360	435	—	412½	- 24 %
8	495	600	540	537½	360	—	630	+ 27 %
9	420	540	510	390	—	—	—	Escaped
10	540	540	480	420	540	—	502½	- 7 %

All healthy on 30th day.

TABLE X.

Padi $22\frac{1}{2}$ gms. + Polished rice 54 gms. daily.

No.	Day, and weight in grammes							Effect of diet and % change in weight by end of exp.	
	1	5	8	10	21	27	30		
11	660	690	600	592½	600	—	615	Beri-beri 28th day. Died 29th day	- 7 %
12	540	570	510	510	540	480	—	Beri-beri 19th day. Died 29th day	- 11 %
13	390	480	420	390	375	—	—	Died suddenly at night 20-21st day	0
14	510	540	510	510	570	—	615	Beri-beri 28th day. Died 29th day	+ 20 %
15	570	570	510	495	555	—	555	Died acute beri-beri 30th day	0
16	630	480	480	435	472½	480	—	Beri-beri 25th day. Died 29th day	- 24 %
17	480	510	450	447½	420	—	420	Beri-beri 26th day. Died 29th day	- 12 %
18	600	570	540	525	600	—	735	Died acute beri-beri 30th day	+ 22 %
19	600	630	600	600	600	—	—	Died suddenly 20th day	0
20	570	540	480	450	480	—	—	Died acute beri-beri 30th day	- 16 %

The birds receiving polished rice as well all developed polyneuritis in from 20 to 30 days, five losing in weight, three remaining constant, and two gaining in weight. The results are set forth in detail in Tables IX and X.

[All these birds showed signs of early beri-beri—dullness, tremors, somnolence, and weakness on 18th to 19th day.]

The amount of anti-neuritic substance contained in 1/20th to 1/25th ration of padi was sufficient to prevent polyneuritis in fowls for at least 45 days, when fed exclusively on the diet (Experiment X), yet, although by the addition of washed polished rice to the diet the amount of active substance was actually slightly increased, the effect of the addition was to produce polyneuritis in less than 30 days. The conclusion clearly emerges that the amount of active substance required by the organism stands in some quantitative relation to the carbohydrate ration which has to be metabolized.

Experiment XII.

In this experiment birds were fed on small and large rations of water-extracted polished rice.

TABLE XI.

Series (a). 1/50th body-weight ration of rice for 1st 5 days, subsequently 1/12th body-weight.

	Effect of diet	% change in wt. by end of exp.
1.	Polyneuritis on 15th day	-18 %
2.	„ „ 15th „	-20 %
3.	„ „ 14th „	-10 %
4.	„ „ 20th „	-40 %
5.	„ „ 15th „	-13 %
6.	Healthy for 24 days	0

Series (b). 1/5th body-weight ration of rice.

1.	Polyneuritis on 11th day	-20 %
2.	„ „ 16th „	-16 %
3.	„ „ 15th „	-27 %
4.	„ „ 14th „	-7 %
5.	„ „ 11th „	-9 %
6.	„ „ 15th „	-3 %

The results show that, unlike what happened in Experiments X and XI, in which the anti-neuritic substance was provided in the diet, there was practically no difference in these two series as to time of onset of polyneuritis. It may be inferred that the lower scale of ration was already sufficient to produce the maximum effect in depletion of

the body store of active substance. Cooper (1913) obtained similar results. It was found that rations of polished rice varying from 1/30th to 1/10th of the body-weight induced polyneuritis in pigeons in about the same time (9 to 26 days), but when the rations fed were below 1/30th polyneuritis was delayed and did not develop until 21 to 33 days had elapsed. The maximum effect is evidently induced by a ration of carbohydrate of about 1/30th the body-weight of the bird. In quantitative investigations of the effect of various conditions upon the rate of onset of polyneuritis it is thus of importance to supply the organism with the minimum amount of vitamine and not to rely merely on the traces of active substance stored in the tissues. This point has frequently been overlooked in the past.

Experiments XIII to XV.

This series of experiments differs from those previously detailed in that all the birds were fed upon polished rice or sago with the addition of a constant amount of dried brewer's yeast sufficient to prevent polyneuritis for from 30 to 50 days when normal rations of carbohydrate were fed. By varying the carbohydrate ration and maintaining the supply of yeast constant, it was thus possible to study quantitatively the effect of increasing the carbohydrate component of the dietary upon the time elapsing before the onset of polyneuritis.

Experiments with pigeons.

The pigeons were first of all fed for three weeks on constant daily rations of whole barley equal to 1/20th their initial body-weight.

Experiment XIII.

Six pigeons were then fed daily on amounts of polished rice and yeast equal respectively to 1/25th and 1/2500th their initial body-weight. For birds weighing 350 gms. the daily quantities of rice and yeast were therefore 14 gms. and 0.14 gm. A second series of pigeons (eight) received daily the same proportion of yeast but two and a half times as much polished rice as the above birds, the rations of rice being thus 1/10th the initial body-weights.

Artificial feeding was employed in all the experiments.

Experiment XIV.

In this experiment the daily rations were as follows :

Series I. (Three birds.) Polished rice = 1/20th initial body-weight.

Yeast .. = 1/3500th " "

Series II. (Three birds.) Polished rice = 1/10th " "

Yeast .. = 1/3500th " "

For pigeons weighing 350 gms. the daily rations were therefore

Polished rice, 17½ and 35 gms. respectively.

Yeast .. 0.1 gm.

TABLE XIII. EXP. XIV.

Series I. Daily ration 1/20th initial body-weight polished rice and 1/3500th body-weight yeast.										
Series II. „ „ 1/10th „ „ „ „ „ „ „ „										
No. of pigeon	Effect of diet upon health of birds	Body-weights							% change in wt. by end of exp.	% change in wt. by 18th day, the average time taken by the birds on large ration to develop polyneuritis
		Initial	Day 5	16	26	37	41	53		
Series I.										
103	Polyneuritis on 37th day	390	390	380	350	300	—	—	- 23 ^o / _o	- 2 ^o / _o
104	Weakness in limbs on 44th day. Polyneuritis on 53rd day	340	320	330	290	250	250	230	- 32 ^o / _o	- 3 ^o / _o
			Day 7	14	21	28	37			
141	Polyneuritis on 32nd day	340	370	330	320	280	290	...	- 15 ^o / _o	- 3 ^o / _o
Series II.										
145	Polyneuritis on 15th day	310	—	370	—	—	—	...	+ 19 ^o / _o	+ 19 ^o / _o
146	Polyneuritis on 16th day	300	370	350	—	—	—	...	+ 16 ^o / _o	+ 16 ^o / _o
148	Weakness in limbs on 22nd day. Polyneuritis on 26th day	320	400	350	320	—	—	...	0	+ 4 ^o / _o

Experiment XV (with chickens).

The birds were first of all fed for three weeks on whole barley, the daily rations being equal to 1/20th their initial body-weight.

Five chickens were then fed on 1/45th their initial body-weight of polished rice and 1/2500th of dried pressed yeast, a chicken of 1450 gms. weight thus receiving daily 32 gms. of polished rice and 0.6 gm. of yeast.

Five other chickens received daily the same rations of rice and yeast as the above, but in addition 1/45th their initial body-weight of sago. Sago was used in this experiment instead of polished rice to facilitate

artificial feeding, as sago passes the crops of birds much more readily than rice¹.

The results of Experiment XIII show that of six pigeons receiving daily $1/25$ th their initial body-weight of polished rice with $1/2500$ th of dried pressed yeast, three remained quite healthy for 95 days and even at the end of that time showed no signs of polyneuritis, one was healthy at the end of 47 days, and two developed polyneuritis in 39 and 52 days. The eight pigeons fed daily on $1/10$ th their body-weight of polished rice with the same amount of yeast as the above birds, however, all developed polyneuritis in from 22 to 46 days.

Experiment XIV shows that pigeons fed on daily rations of polished rice equal to $1/20$ th their initial body-weight with $1/3500$ th of yeast developed polyneuritis in from 32 to 44 days, but those fed on $1/10$ th their body-weight of rice with the same amount of yeast became ill in from 15 to 22 days.

Pigeons fed exclusively on the same polished rice in amounts equal to $1/10$ th to $1/30$ th their initial body-weight daily developed polyneuritis in from 9 to 26 days (Cooper, 1913). It is thus seen that, while the addition of a small amount of yeast to the polished rice diet could prevent polyneuritis in pigeons for a considerable time when the daily ration of rice did not exceed $1/20$ th the initial body-weight, when the ration of rice was increased to $1/10$ th, the yeast had little protective effect and the period elapsing before the onset of polyneuritis did not greatly exceed that elapsing when the diet was exclusively polished rice.

Experiment XV (with chickens) shows that of the five birds fed daily on $1/45$ th their initial body-weight of polished rice with 0.5 to 0.7 gm. of yeast one was still healthy on the 100th day of the experiment, three developed polyneuritis on the 94th to 97th days, and one on the 62nd day. The other five birds receiving in addition $1/45$ th their initial body-weight of sago daily fell ill with polyneuritis in from 13 to 31 days. As in the case of the previous experiments with pigeons, doubling the daily ration of carbohydrate thus greatly accelerated the development of polyneuritis.

The eight pigeons (Experiments XIII and XIV) fed on the small rations all lost in weight, while of the 11 pigeons fed on large rations

¹ It should be mentioned that during the first week of the experiment the rations of carbohydrate employed were somewhat larger, being $1/30$ th and $1/15$ th the initial body-weight of the birds. As some difficulty was met with in feeding these large amounts, the rations finally employed were as above.

(1/10th their body-weight) four gained in weight, three remained constant in weight, and the remaining four lost to as great an extent as some of the birds fed on the small rations and yet developed polyneuritis earlier. These results are of interest as they indicate that a dietary deficient in the anti-neuritic substance can sometimes maintain body-weight and even lead to increase in weight. Similarly, in Experiment XV of the five chickens fed on the large ration, three maintained their body-weight, but two lost in weight to as great an extent as the birds fed on the small ration and again developed polyneuritis considerably earlier.

The results thus confirm the earlier observations that the amount of anti-neuritic material required by the organism is not constant and independent of the quantity of food provided, but as the ration of carbohydrate is increased the demand of the organism for the active substance is also increased and if this be not adequately met, polyneuritis follows. When the carbohydrate ration was doubled (as in Experiment XV) by the addition of sago to a diet consisting of polished rice and yeast, the development of polyneuritis was accelerated considerably more than two-fold. Thus, when daily rations of polished rice and yeast equal respectively to 1/45th and 1/2500th the initial body-weight of the birds were fed, polyneuritis did not occur for nearly 100 days, but when the diet was supplemented by an equal quantity of sago, symptoms of polyneuritis appeared in from 13 to 31 days.

In this experiment the excreta of the fowls were collected daily, dried at ordinary temperatures by means of an electric fan, and the amount of undigested starch estimated by hydrolysis with acid and determination of the quantity of sugar produced by reduction (Bertrand) and polarimetric methods. The results, given in Table XV, were obtained.

The results show that in the case of the birds fed on the small rations the percentage of starch digested varied from 95 to 100 per cent., and in the case of the birds fed on the large rations from 90 to 100 per cent., the greater part of the starch fed thus being hydrolysed in the alimentary canal. Only small amounts of sugar could be detected in the excreta, and no dextrin was found at all.

Cooper (1914) found that alcoholic extracts of the excreta of a chicken fed on grain and the faeces of a rabbit fed on cabbage cured polyneuritis in pigeons, so that a possible explanation of the effect of large rations of starch in accelerating polyneuritis was that a portion of the starch was not digested, and interfered with the absorption of the

TABLE XV.

	Bird	Period during exp.	Starch fed	Starch excreted	Period during exp.	Starch fed	Starch excreted
Small rations	A	5th-11th day	204	5.0	12th-18th day	156	2.5
					19th-25th „	156	0
	B	„	228	2.5	—	—	—
	C	„	228	7.0	12th-18th „	162	9.0
					19th-29th „	250	6.0
Large rations	D	„	228	1.0	12th-25th „	325	4.0
	E	„	207	2.0	12th-33rd „	492	0
	F	„	346	4.5	—	—	—
	G	„	413	7.0	12th-33rd „	965	5.0
	H	„	476	8.0	12th-25th „	674	0
	I	„	325	3.5	12th-18th „	351	34.0
					19th-25th „	351	16.0
	K	„	636	3.1	12th-18th „	468	36.0
					19th-29th „	720	8.0

anti-neuritic substance supplied in the diet. As however the amount of starch excreted by the fowls in Experiment XV was so small, it seemed improbable that the above explanation could hold at any rate for the particular rations employed in that experiment. Nevertheless it was decided to attempt to ascertain whether the anti-neuritic substance could be detected in the excreta. Pigeons were fed daily on 1/25th their body-weight of polished rice to which was added varying amounts of the pulverised dry excreta of chickens A, E (small rations of rice) and G, I (large rations).

Assuming only half the amount of active substance fed to the chickens was absorbed, the amount contained in the excreta administered should have prevented polyneuritis in the pigeons for 39 to 95 days (see Experiment XIII).

Experiment XVI.

TABLE XVI.

Pigeon	Amount of dry excreta fed daily	Effect on pigeon
1	3.5 g. of chicken A (small ration)	Weakness in wings and limbs 25th day. Polyneuritis acute 32nd day.
2	1.5 g. of chicken E (small ration)	Polyneuritis on 22nd day.
3	3.5 g. of chicken G (large ration)	Weakness in wings and limbs on 27th day.
4	4.5 g. of chicken I (large ration)	Weakness in wings and limbs 25th day. Acute polyneuritis 32nd day.
Pigeons fed on 1/25th body-weight of polished rice only		Polyneuritis in 20-28 days.

The excreta were evidently free from the anti-neuritic substance, and it thus seems that in Experiment XV the rapid onset of polyneuritis in the case of the birds receiving the large ration of carbohydrate was not due to imperfect absorption of the active material.

Experiments were next carried out with the object of ascertaining (1) whether the active substance is adsorbed by starch and (2) whether chickens digest the starch as readily when the ration is raised to 1/10th their body-weight (the maximum ration employed in the above digestion experiment being 1/20th the body-weight).

To 50 c.c. of a curative yeast extract 20 gms. of potato starch were added, and the mixture was allowed to stand at room temperature with frequent shaking for several days. The minimum curative dose of the solution for neuritic pigeons of about 300 gms. weight was determined before and after the addition of the starch, 10 animals being employed altogether. It was found that 2-3 c.c. of the solution before and after treatment with starch was just sufficient to bring about complete recovery, smaller doses having only a slight ameliorative effect. There was thus no evidence that starch adsorbed the active substance to any appreciable degree.

It seemed still possible, however, that with a much larger ration of starch the absorption of the active substances from the gut might be interfered with.

Four chickens were therefore fed on very large rations of polished rice (1/10th their body-weight) with the addition daily of 4, 6, 2½, and 1½ gms. of dried pressed brewer's yeast respectively. The excreta were collected daily, dried at 20° C. by means of an electric fan, and ground to a fine powder. The birds developed polyneuritis in the remarkably short period of 10 days. The amount of starch in the excreta was estimated and we were surprised to find that of this enormous ration the birds had digested 93 to 98 per cent., the amount of starch excreted per diem varying from 2 to 8 gms. Four pigeons were fed on rations of polished rice equal to 1/25th the body-weight and fixed quantities of the excreta. The amounts given daily were such that assuming provisionally that one half the yeast fed to the original chicken was excreted, they should have contained sufficient yeast (0.15 gm.) to prevent polyneuritis in the pigeons for from 39 to 95 days. (See Experiment XIII.) The pigeons however all developed polyneuritis in from 21 to 30 days, while control birds fed on the same ration of polished rice without the addition of excreta fell ill in from 20 to 28 days.

The addition of the excreta to the rice diet thus neither delayed nor

prevented polyneuritis in the pigeons, and this shows that the rapid onset of polyneuritis which results from the consumption of diets containing large rations of polished rice is not due to interference with the absorption of the anti-neuritic substance.

The main conclusion to be drawn from these experiments is therefore that the amount of anti-neuritic substance required by the organism increases with the quantity of carbohydrate *metabolized*. For the maintenance of health the intake of active substance must therefore be adjusted, as so to stand in some quantitative relation to the amount of carbohydrate included in the diet.

This fact is of practical importance in the prevention of beri-beri, as attention must evidently be paid not only to the absolute amount of anti-neuritic foodstuff incorporated into the dietary, but also to the proportion which this bears to the total carbohydrate ration. The exact ratio which must be maintained has not yet been determined, but the results so far obtained show that when the carbohydrate ration was doubled polyneuritis was induced more than twice as rapidly. Evidently then for the prevention of beri-beri the proportion of the anti-neuritic foodstuff in the diet must be maintained as high as possible, and large rations of foodstuffs deficient in the active substance carefully avoided.

Some minor points arising out of the results deserve mention. It was suggested previously (Cooper, 1914) that the curative action of extracts of excreta was due in some degree to the capacity of bacteria growing in the intestine to synthesize the anti-neuritic substance. Extracts of 2 gms. of *B. coli* (dry) however had no effect upon neuritic pigeons, but this could not be regarded as conclusive evidence, as so small a weight of bacilli was used. As, however, excreta are very rich in bacteria, the failure of as much as 1.0 to 5.0 gms. of excreta daily to prevent or even delay polyneuritis in pigeons fed on polished rice suggests that bacteria compared with yeast (0.15 gm. daily of which prevents polyneuritis for 39 to 95 days) contain an insignificant amount of anti-neuritic substance.

The results also show that birds can digest enormous rations of starch, amounting daily to 1/10th their body-weight.

SUMMARY.

(1) Chickens fed on 1/20th their body-weight daily of padi, cured or parboiled unpolished rice, or fresh (partly polished) rice, remain free from polyneuritis for at least 28 to 100 days.

(2) Chickens fed on the same ration of polished Siam rice develop symptoms of polyneuritis in from 20 to 70 days.

(3) When the polished rice is soaked in excess of water for 24 hours the birds succumb to polyneuritis in from 10 to 35 days ; when the rice is soaked for 48 hours the disease appears still more readily, viz. in 15 days.

(4) Chickens fed on 1/20th their body-weight of parboiled rice which had been soaked for 24 hours also develop polyneuritis in from 13 to 39 days. This fact is of practical importance, as epidemics of beri-beri have been traced to the practice of soaking unpolished rice prior to cooking and discarding the water.

(5) Although birds fed on diets of polished rice, sago, or glucose develop polyneuritis, when fed on commercial starch they often fail to do so and merely lose considerably in weight. An explanation is offered.

(6) While fowls fed on 1/20th their body-weight of padi remain free from polyneuritis for at least 60 to 100 days, when this ration is supplemented by 1/6th the body-weight of washed unpolished rice or 1/10th of polished rice polyneuritis appears in from 20 to 30 days.

(7) On the other hand, chickens fed on rations varying from 1/5th to 1/30th of their body-weight of washed unpolished rice develop polyneuritis in about the same time, 10 to 20 days.

(8) On dietaries composed of rations of polished rice varying from 1/20th to 1/40th the body-weight and of yeast varying from 1/2500th to 1/3500th the body-weight pigeons and chickens do not develop polyneuritis until at least 32 to 100 days have elapsed, but when the carbohydrate ration is doubled by the addition of polished rice or sago the birds fall ill in from 13 to 46 days.

(9) Even when daily rations of polished rice as large as 1/10th the body-weight are fed to chickens together with varying amounts of yeast, 93 to 98 per cent. of the carbohydrate is digested and absorbed and the excreta contain no anti-neuritic substance. This shows that the rapid development of polyneuritis induced by feeding large rations of starch is not due to interference of undigested carbohydrate with the absorption of the active material.

(10) Intestinal bacteria, unlike yeast, contain little anti-neuritic substance.

Conclusion and practical application of the results.

The amount of anti-neuritic substance required by the organism increases with the quantity of carbohydrate metabolized. For the maintenance of health the intake of active substance must therefore

be adjusted, so as to stand in some quantitative relation to the ration of carbohydrate ingested, and it is when this necessary balance is not maintained in the dietary that beri-beri results. Although as ordinarily induced beri-beri can be described not inaccurately as a "deficiency disease," it may thus actually develop when a dietary containing an adequate amount of the anti-neuritic substance is regularly supplemented by a ration of a carbohydrate foodstuff deficient in this essential substance.

This is obviously of great practical importance in the prevention of beri-beri. Attention must be paid not only to the absolute amount of anti-neuritic foodstuff incorporated in the dietary, but to the proportion which this bears to the total carbohydrate ration. The precise relation which must subsist between the supply of active material and the amount of carbohydrate metabolized has not yet been ascertained, nor has it been determined for the other normal components of a dietary, *e.g.* protein and fat. It is therefore advisable to maintain the proportion of anti-neuritic foodstuff in the diet as high as possible, and large rations of foodstuffs deficient in the essential substance should be carefully avoided.

In the preparation of a dietary to obviate beri-beri, it thus becomes necessary to consider not merely its absolute content of anti-neuritic material, but also its total calorific value.

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SEROLOGICAL TESTS.

- I. ON THE PERSISTENCE OF PRECIPITINS IN SERA STORED *IN VITRO*.
- II. ON THE REACTIONS OBTAINED WITH (a) COMPLEMENT FIXATION TESTS AND (b) PRECIPITIN TESTS, WITH THE GUT CONTENTS OF BLOOD-SUCKING ARTHROPODS.

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I. *The persistence of precipitins in vitro.*

VARIOUS authors have noted that antisera sealed in glass tubes may preserve their properties for considerable periods. Thus Nuttall (1904) found that definite reactions could be obtained with antisera that had been kept for fourteen months, and it seemed probable that reactions could be obtained with much older antisera.

During his experiments Prof. Nuttall prepared many kinds of antisera by injecting rabbits with the blood or serum of different species of animals. These antisera, and also their homologous bloods, were stored in sealed glass tubes, and have been kept together in a dark case in the laboratory, at a temperature of about 15° C. Some of the bloods, moreover, were preserved dried on filter paper. Prof. Nuttall kindly placed this material at our disposal and suggested that it would be of interest to determine whether the antisera still preserved their properties.

The results are given in detail in Table I and it will be noted that in two cases well-marked specific reactions were obtained. In others, however, the antisera also reacted with related species, whilst occasionally somewhat anomalous results were obtained. Thus hyaena antiserum gave a distinct precipitum with dog blood as well as hyaena, and a slight precipitum with blood from a Ross seal. Similarly dog

antisera reacted with raccoon, as well as dog blood; and Mexican deer antiserum with Mexican deer, antelope, wild sheep, and goat blood. Specific reactions were obtained only with orang-outang antiserum, prepared 17. XI. 1902, and hippopotamus antiserum, prepared 11. III. 1903. On the other hand the following antisera gave entirely negative reactions: man, ox, cat, llama, seal, and hedgehog.

The tests were made with 1 to 100 dilutions of the antigens. To 0.5 c.c. of each dilution was added 0.05 c.c. of the antiserum.

II. *The reactions obtained with (a) Complement fixation tests and (b) Precipitin tests, on the gut contents of blood-sucking arthropods.*

It is well known that the properties of the precipitinogen (blood albuminoids), in the dried state, may be preserved for years and still give specific reactions, but it has been found that in the process of digestion in vertebrates, the properties are rapidly lost. On the other hand, in blood-sucking arthropods digestion proceeds much more slowly, and it has been found possible, by means of the precipitin test, to recognise the species of animal on which they have been feeding. Having a number of ticks in the laboratory, that had been starved for periods varying up to two years, it was decided to examine their gut contents by the complement fixation and precipitation tests.

(a) Complement fixation tests.

The following technique has been employed for these experiments.

The haemolysin¹ was tested by mixing varying amounts (1 c.c. of a 1:100, 1:200, 1:400 dilution) with 1 c.c. of a 5% emulsion of red blood corpuscles of sheep, and 1 c.c. of a 1:10 dilution of guinea-pig serum (as complement). The mixture was then made up to 5 c.c. by adding 0.9% salt solution and placed for one hour in a thermostat at 37° C. Complete haemolysis was obtained with 1 c.c. of a 1:300 dilution of the haemolysin.

The following is an example of one of the tests:

Haemolysin	1 c.c. (1:50 dilution)	1 c.c. (1:100)	1 c.c. (1:200)	1 c.c. (1:300)	1 c.c. (1:400)	1 c.c. (1:800)
Complement (1:10 dilution)	1 c.c.	1 c.c.	1 c.c.	1 c.c.	1 c.c.	1 c.c.
5% sheep red blood corpuscles	1 c.c.	1 c.c.	1 c.c.	1 c.c.	1 c.c.	1 c.c.
0.9% NaCl sol.	2 c.c.	2 c.c.	2 c.c.	2 c.c.	2 c.c.	2 c.c.
Haemolysis	Complete	Complete	Complete	Complete	Partial	Absent

¹ Rabbit anti-sheep blood serum.

When making a reaction two or three times as much haemolysin was employed as was necessary to cause complete haemolysis. The haemolysin was only tested once every two or three weeks, as it changes very slowly, but the complement had to be tested before each experiment.

The complement was tested in a similar manner to the haemolysin, employing varying amounts of complement and a fixed amount of haemolysin, as shown in the following table:

Complement (1:10 dilution)	0.2 c.c.	0.4 c.c.	0.6 c.c.	0.8 c.c.	1.0 c.c.	1.2 c.c.
Haemolysin (1:100 dilution)	1 c.c.	1 c.c.	1 c.c.	1 c.c.	1 c.c.	1 c.c.
5 % sheep r.b.c.	1 c.c.	1 c.c.	1 c.c.	1 c.c.	1 c.c.	1 c.c.
0.9 % NaCl sol.	2.8 c.c.	2.6 c.c.	2.4 c.c.	2.2 c.c.	2.0 c.c.	1.8 c.c.
Haemolysis	Absent	Partial	Complete	Complete	Complete	Complete

As in the case of the haemolysin, we always employed two or three times as much complement as was required to bring about complete haemolysis.

Since the serum containing anti-bodies, or the substance which contains the antigen, is able of itself to fix complement in larger or smaller amounts (non-specific fixation), before testing it is necessary to determine the largest quantity of these which alone will not fix complement. For the test half this quantity was employed. The following table is an example of such a test:

Anti-fowl ¹ serum	0.04 c.c.	0.06 c.c.	0.1 c.c.	0.2 c.c.	0.4 c.c.
Complement (1:10 dilution)	1 c.c.	1 c.c.	1 c.c.	1 c.c.	1 c.c.
0.9 % NaCl sol.	1.96 c.c.	1.94 c.c.	1.9 c.c.	1.8 c.c.	1.6 c.c.

These mixtures are placed for one hour in a thermostat at 37° C. and the following then added:

5 % sheep r.b.c.	1 c.c.	1 c.c.	1 c.c.	1 c.c.	1 c.c.
Haemolysin (1:100 dilution)	1 c.c.	1 c.c.	1 c.c.	1 c.c.	1 c.c.
Haemolysis	Complete	Complete	Complete	Absent	Absent

Having by these preliminary examinations determined the appropriate amounts of haemolysin, complement and anti-body, we were then in a position to carry out our experiments. The gut contents of the following arthropods were examined:

¹ Inactivated by heating to 56° C. for half an hour.

- (1) *Argas persicus* fed on fowl 5 days previously (kept at 30° C.).
- (2) 1 month .. (..).
- (3) 4 months .. (..).
- (4) 11½ (..).
- (5) 23 (kept at 10° C.).
- (6) *Ornithodoros moubata* fed on fowl 12 months previously (kept at 30° C.).
- (7) 12 and also fed on mouse
6 months previously (kept at 30° C.).
- (8) *Pediculus vestimenti* fed on man 3 days previously (kept at 30° C.).

In all these cases total fixation of the complement (no haemolysis) was obtained with the corresponding antisera, whilst invariably in the controls haemolysis was complete.

The following is an example of one of our experiments :

Extract of <i>Argas</i> fed on fowl 11 months previously	0.2 c.c.	0.4 c.c.	0.2 c.c.	0.4 c.c.	0.2 c.c.	0.4 c.c.
Anti-human serum	—	—	0.05 c.c.	0.05 c.c.	—	—
Anti-fowl serum	—	—	—	—	0.05 c.c.	0.05 c.c.
Complement (1:10 dilution)	1 c.c.	1 c.c.	1 c.c.	1 c.c.	1 c.c.	1 c.c.
0.9 % NaCl solution	1.8 c.c.	1.6 c.c.	1.75 c.c.	1.55 c.c.	1.75 c.c.	1.55 c.c.

Placed for one hour at 37° C. and then added :

5 % sheep r.b.c.	1 c.c.	1 c.c.	1 c.c.	1 c.c.	1 c.c.	1 c.c.
Haemolysin (1:100 dilution)	1 c.c.	1 c.c.	1 c.c.	1 c.c.	1 c.c.	1 c.c.
Haemolysis	Complete	Complete	Complete	Complete	Absent	Absent

(b) Precipitin tests.

Having obtained positive results with the complement fixation method in every case, we decided to try also the precipitin test, using as fowl antiserum one which was able to produce a precipitum with a 1:32000 dilution of fowl serum, and as human antiserum one which produced a precipitum with a 1:30000 dilution of its corresponding antigen.

In every case after standing for ten minutes a well-marked cloud appeared, and after 24 hours a distinct precipitum, the controls being negative.

From the results of these two series of experiments it is obvious, therefore, that the process of digestion takes place extremely slowly in ticks, and that the digestive fluids do not affect the albuminoid substances which give complement fixation and precipitin reactions. As might be expected a mixture of bloods inside the gut of the tick does not affect the reactions, for *Ornithodoros moubata* that had been

fed on a fowl and subsequently on a mouse gave as definite reactions as other ticks which had been fed only on fowls. In *Pediculus vestimenti* the digestion of the blood takes place much more rapidly than in ticks, but well-marked reactions were obtained in the case of lice that had been fed three days previously. At a temperature of 30° C. these insects could not endure starvation for any longer period.

The results of both series of experiments seemed to be so surprising as to be worth recording. The methods we have employed may be applied practically in the identification of the sources of food of blood-sucking arthropods. The preservation of the properties of blood for as long as 23 months in the gut of a tick raises an interesting question in physiology.

SUMMARY OF RESULTS.

1. Sera and antisera when kept in the dark at room temperature sealed in glass tubes occasionally give specific reactions after a period of 12 years. In most cases, however, negative results were obtained with the antisera, whilst in others the reactions were non-specific.

2. Fowl blood will still give specific complement fixation and precipitin reactions after being in the alimentary canal of either *Argas persicus* or *Ornithodoros moubata* for at least 23 months. The gut contents of *Pediculus vestimenti* fed on man three days previously, also will give specific reactions with human antisera.

Note. The human and fowl antisera used in the second series of experiments were obtained from the Bacteriological Department of the University of Budapest. The sheep red blood corpuscles were isolated from blood purchased at the slaughter-house, whilst the complement was obtained by killing a guinea-pig and taking the blood from its heart. The haemolysin was purchased.

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THE THERMOPRECIPITIN METHOD IN THE DIAGNOSIS OF BUBONIC PLAGUE IN CADAVERS.

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THE diagnosis of bubonic plague in rat cadavers, the examination of which is a recognised method of prophylaxis for ships or other communities, which may have been in contact with endemic centres, is attended by especial difficulties, and, on the other hand, calls for especial speed and finality in technique.

It rests at present finally on the isolation and identification by the serum reaction (agglutination) of the *B. pestis*. Whether the organism is cultivated directly from the cadaver or indirectly by interposition of an experimental animal, this requires a delay of at least two days, in a matter in which time is of the highest importance, as, for instance, when a suspected ship is detained pending a decision. But for agglutination a pure culture is necessary, and to obtain this is a matter of difficulty owing to the speed with which *B. pestis* is overgrown at its optimum growth temperatures by bacteria of intestinal and saprophytic origin. The difficulty becomes an impossibility when, as often happens, the cadavers arrive at the laboratory in a state of decomposition, especially in hot climates, *B. pestis* having very little resistance to high temperatures (Dunbar and Kister, Kister and Schumacher).

On these considerations any diagnostic aid which would obviate the necessity of isolation and pure cultivation of *B. pestis*, would be welcome. Such a diagnostic method is the "Thermoprecipitin Method" devised by Ascoli for the detection of anthrax in like circumstances.

This research is an attempt to discover how far it may be of use in the especial case of bubonic plague. The experiments were already

well advanced, when the work of Piras, on the same subject, appeared in the *Centralblatt für Bakteriologie*, Sept. 1913, and were thereafter continued on the same lines on which they had been begun. Piras' results are discussed below.

Ascoli's method consists in bringing together anthrax serum of high value and decoctions of the suspected tissues, the occurrence of precipitation being taken as proof of the presence of anthrax. The decoctions are prepared by emulsifying the tissues in normal salt solution, heating for some minutes in the water-bath at boiling temperature, and filtering. This process decolorises and eliminates coagulable proteid from the extract, without influencing adversely the final reaction, since the bacterial antigens concerned are known to have a very high resistance against heat (Nicolle, Pick). Ascoli proved that :

(1) Extracts of anthrax-affected organs gave a precipitate similar to that given by extracts of *B. anthracis*, when brought in contact with anthrax-immune serum of high value.

(2) This precipitate was specific ;

(3) and was still demonstrable in organs in which, owing to more or less advanced putrefaction, the usual bacteriological methods were unavailable.

(4) The reaction might give a negative result, though the case had actually been one of anthrax, when the number of bacilli in the organs at death had been inconsiderable—a conclusion to be expected on *a priori* grounds.

Technique. The technique followed the lines laid down by Ascoli. The organs (heart, spleen, liver, and—where found—diseased gland, as being those in which the bacteria occur in greatest number), were, after appropriate bacteriological examination, by means of film preparations, cultures, and inoculation of experimental animal, removed from the cadaver, minced with the scissors, and then gently shaken up with sterile normal salt solution in the proportion of 4–5 c.c. solution to 1 gramme of organ. The flask containing the suspension was plunged into boiling water for five minutes, the suspension allowed to cool, filtered through doubled filter-paper, if necessary, several times, till quite clear or at most slightly opalescent, and the filtrate used for the precipitation experiment. This was carried out by the "ring" or "Schichtungsmethode" of Ascoli, viz. a few drops (c. 0.25 c.c.) were transferred to a small glass tube, and a corresponding volume of serum introduced by means of a glass pipette drawn out into a capillary tube, the capillary end being brought to the bottom of the tube, before the

serum was allowed to flow out. The two fluids thus remained in two distinct layers, the heavier serum below. The presence of specific antigen in the extract, *i.e.* a positive reaction, was indicated by the appearance of a whitish ring at the surface of contact. The glass tubes were 7 cms. deep by 4.5 mm. diameter, and were made, according to the experience of Meyer, with flat instead of rounded bottoms, in order to eliminate the optical disturbance caused by refraction from a curved surface. They could easily be cleaned under a small jet of cold water, and so used again. The tubes were mounted on a black stand to the back of which an adjustable black screen was affixed. The rats used were the ordinary white laboratory species. They were infected by subcutaneous injection of $\frac{1}{5}$ to $\frac{1}{10}$ platinum loop of a 24 to 48 hour agar slope culture, and succumbed on the 3rd to 5th day. The strains of *B. pestis* were two which had been cultivated in the Institute for more than ten years, and were those used in the production of the "Bernier" polyvalent pest serum. One strain originated from St Petersburg, the other from the laboratory of Prof. Schottelius in Freiburg-in-Breisgau. "Bernier" pest serum, prepared in the Institute according to Yersin's method, or a mixture of "Bernier" and Cronstadt sera, was employed. Details of technique, which differed in different parts of the work, are given with the corresponding table.

The specificity of the reaction. Kraus found that plague-immune serum caused a precipitate in extracts of *B. pestis*, but not of *B. typhi*, nor of the cholera vibrio; Zlatogoroff that the reaction was negative also in the case of the bacteria most nearly allied in morphology and pathogenicity to the *B. pestis*, viz. *B. gallinarum* of fowl-cholera, *B. suis* of swine-fever, *B. septicaemiae* (Koch, Gaffky), and *B. pseudotuberculosis rodentium* of Pfeiffer. McConkey, on the other hand, states that the last-named causes a precipitate, though quantitatively less than the specific, in plague serum.

Kolle and Otto examined 50, Zlatogoroff 22, strains of *B. pestis* from epidemics all over the world, and found that culture extracts of each gave a marked reaction with the sera used by them.

The results of the present author's experiments with these and other bacteria are incorporated in Table A. In the case of each disease the precipitation experiment was carried out with (a) extracts, prepared as described above, from the fresh organs, after bacteriological examination had shown the presence therein of the causal bacteria in great numbers; and (b) filtrates from suspensions in normal salt solution of the bacteria.

TABLE A.

Bacterium	Bacterial Extract		Organ Extract	
	Normal Serum	Plague Serum	Normal Serum	Plague Serum
Normal organs			-	-
<i>B. pestis</i> (1)	-	+++	-	+++
„ (2)	-	+++	-	+
<i>B. coli murium</i> (isolated from normal rat faeces)	-	+		
Saprophyte isolated from cadavers	-	+		
<i>B. gallinarum</i> :				
(1) from Kralsehe Museum, Vienna	-	±		
(2) from L. W. Gans, Frankfort.. ..	-	±		
<i>B. suisepicus</i> :				
(1) from Kral. Mus., Vienna	-	±	-	±
(2) from L. W. Gans, Frankfort.. ..	-	±	-	±
<i>B. dysenteriae</i>	-	±	-	-
<i>B. typh.</i>	-	+	-	-
<i>Diplococcus pneumoniae</i>	-	±	-	-
<i>B. paratyphosus</i> B ..	-	±	-	-
<i>B. paratyph.</i> Dansyz ..	-	-	-	-
<i>B. pseudotuberculosis ro-</i> <i>dentium</i> of Pfeiffer	-	±	-	-

The bacterial filtrates were prepared as follows: A 48-hour agar slope culture was emulsified in 6 c.c. of normal salt solution, mechanically shaken for two hours, plunged into boiling water for five minutes, and finally filtered through a porcelain column. In the case of *B. pestis*, Kraus' experience that filtrates from old (14-30 days) bouillon cultures give an intense reaction, was confirmed, but bouillon was rejected as an extractive medium, because the specific gravity of bouillon plus bacterial extractives may be so near that of serum that a sharp surface of contact is difficult to obtain.

With each experiment a control was made with normal serum and the filtrate concerned. The plague serum was tested from time to time with a plague bacterial extract of known value.

The experiments were carried out at room temperature (18°-22° C.), and the results kept under observation for 20 minutes, and then noted again after having stood overnight, *i.e.* for about 16 hours. The degrees of the positive reaction are indicated in the table as follows:

- + + + = flocculent ring within 15 minutes, deposit in 16 hours,
- + + = well-marked ring without distinct flocculi, in 15 minutes, deposit in 16 hours.
- + = cloud in 15 minutes, distinct deposit in 16 hours.
- ± = cloud in 15 minutes, no deposit in 16 hours.
- = no cloud, no deposit.

In the case of *B. pestis* extracts, the reaction always appeared immediately as a bluish-white ring at the surface of contact, waxed steadily in intensity during the first 15 to 20 minutes, during which the experiment was under constant observation, and, after 16 hours, showed itself as a flocculent deposit at the bottom of the tube, the disturbed surface of contact above showing a secondary diffuse flocculent precipitation-ring. No reaction was observed with normal serum and plague extracts within 20 minutes, though a faint turbidity was noticed once or twice after 16 hours. Normal salt solution, *i.e.* the medium used for extraction, in contact with plague-immune serum, was likewise consistently negative.

The unspecific reactions may be eliminated by dilution of either extract or serum. Two different *B. pestis* extracts still reacted with serum at a dilution of $\frac{1}{2000}$, whereas the extracts of *B. coli*, *B. gallinarum*, and a saprophyte isolated from a cadaver, failed to react with serum at a dilution of $\frac{1}{2}$, the positive reaction being taken as the appearance of a ring or cloud at the surface of contact within 15 minutes.

The reaction in decomposing cadavers. The experiments are in three series, in each of which the treatment of the cadaver after death differed.

Series I. The cadavers were put unopened into glass jars open to the atmosphere through a wire-gauze lid, were covered with moist earth, and were kept in a cellar, at a temperature which varied between 10° and 18° C. At intervals a cadaver was removed and opened under precautions against introduction of foreign organisms. A guinea-pig was infected with material from liver and spleen. The method of infection of the guinea-pig is indicated in each case in the table.

The results are incorporated in Table B.

From these seven preliminary experiments appear the facts (1) that, although the cadaver, in which decomposition was most advanced, and which yet contained living virulent *B. pestis*, gave no precipitation ring, a 29 days' old cadaver reacted strongly positive, while two 10 days' old were negative; and (2) that all three positive precipitation results coincided with positive inoculation experiments. A possible explanation of both observations lay in the bacterial content of the

TABLE B.

Experiment	Days in earth	Degree of decomposition	Smear preparations	Method of inoculation of guinea-pig	Result of inoculation	Cause of death	Precipitation experiment
1	8	Slight	Numerous saprophytes ? Plague B +	Subcutaneous injection of bouillon emulsion spleen and liver	✕ 5th day	Plague +	+ +
2	8	„	Numerous saprophytes Plague B +	„ „	✕ 6th day	Plague +	+ + +
3	10	„	? Plague B + ?	„ „	Still alive and healthy 26th day	Plague -	-
4	10	„	-	Inunction of shaved abdominal skin with spleen and liver	Still alive and healthy 26th day	Plague -	-
5	32	Moderate	-	Pea-sized piece of liver and spleen in abdominal skin pouch	✕ 7th day	Plague +	+ + +
6	39	„	-	„ „	Alive and healthy 21st day	Plague -	-
7	68	Advanced	-	„ „	✕ 4th day	Plague +	-

organs at death. Where that had been considerable, precipitation and inoculation would both tend to a positive; where inconsiderable, to a negative result.

Series II. Accordingly, in the next series a rough estimate was made of the comparative number of plague bacilli in the heart-blood of each cadaver at death. As soon after death as possible, the heart was exposed by a small incision through the chest wall, opened, and several films made from the blood. The bacilli in a number of microscopic fields of each film were counted, and the average reckoned. The results are entered thus: *Few* = less than 3, *considerable* = between 3 and 10, *numerous* = more than 10 bacilli in one field of the oil immersion lens. As will be seen from Table D, which shows the comparative bacterial contents of blood, bubo, spleen and liver in 12 fresh cadavers, a correspondence does exist between the number of bacteria in the circulation and that in the spleen and liver. This examination of the fresh cadaver necessitated its being dipped into absolute alcohol for a few minutes before being opened, in order to kill any living fleas which might be in the fur. The alcohol was afterwards allowed to completely evaporate before the cadavers were stored away. In this series they were kept as before in glass jars with wire-gauze lids, but they were covered with dry grain, and the temperature of the storage room was 28°-30° C.

After the first two experiments the remaining cadavers were allowed to freeze in the open at a temperature of $-6^{\circ}\text{C}.$ and then returned to the incubator. The object was to kill off the plague bacteria, the resistance of which to variations of temperature is known to be low, though that to constant low temperatures is high. In all the smear preparations made direct from the organs, numerous saprophytes were seen, but fewer than in the films from Series I. The cultural examination was carried out on the lines of the practice in the Hamburg Hygienic Institute: from each organ a bouillon and an agar-plate medium were inoculated, the former by introduction of a small piece of organ, the latter by gently drawing across it the fresh cut surface of the organ. The bouillon was incubated at $30^{\circ}\text{C}.$ and examined after 24 hours. If it contained polar-staining Gram-negative organisms, a second series of agar plates was inoculated from it. Both series of agar plates were kept in the ice chest at $+6^{\circ}\text{C}.$, and showed the first colonies in five to seven days, *i.e.* in the same time as *B. pestis* under the same conditions. All the cultures were negative, the agglutination test with plague serum being applied to all doubtful organisms.

All the experimental inoculations were carried out by the abdominal skin-pouch method.

In the description of the degree of decomposition of the cadaver, "*moderate*" = strong smell of decomposition, organs easy to recognise; "*advanced*" = organs recognised with difficulty. *Bubo*, though searched

TABLE C.

Experiment	Bacteria in blood at death	No. of days between \pm and experiment	Degree of decomposition	Smear preparations	Cultures	Result of inoculation of guinea-pig	Result of precipitation experiment
1	Few	8	Slight	-	-	+	+
2	Numerous	15	Moderate	-	-	\times on 2nd day	+
3	Few	21	"	-	-	-	+
4	Numerous	21	"	-	-	-	+
5	Numerous	25	Advanced	-	-	-	+
6	None	25	"	-	-	-	+
7	Few	28	Moderate	-	-	-	+
8	None	28	"	-	-	-	+
9	Considerable	31	"	-	-	-	+
10	Few	31	"	-	-	-	+
11	Few	39	"	-	-	-	+
12	None	39	"	-	-	-	+
13	Numerous	43	"	-	-	-	+
14	Considerable	43	"	-	-	-	+
15	Few	45	Advanced	-	-	-	+
16	Considerable	45	"	-	-	-	+
17	Few	49	"	-	-	-	+
18	Numerous	49	"	-	-	-	+
19	Considerable	49	"	-	-	-	+

for, was never recognised, although the rats had received the same subcutaneous dose as those in Table D, where bubo was found in 11 out of 12 cases. This is in accordance with Zlatogoroff's experience that the bubo is the first organ to disappear, as the result of decomposition, the spleen being the next to follow.

Of the 19 cases, 13—*i.e.* well over half—gave a certain positive precipitation, one a doubtful, and five a completely negative result. Between the result and the degree of putrefaction of the cadaver no sort of correspondence appears, whereas that between the result and the bacterial content of the blood seems as exact as the method of determination of the latter can permit. Thus, the five negative cases occur where the bacteria were "*few*" or none, the six most strongly positive where the latter were "*considerable*" or "*numerous*," and in none of the four cases where "*few*" bacteria correspond to a positive result, does that result reach more than a + degree.

Series III. The procedure after death was the same as in II, but the cadavers had been left so long in the incubator that all were completely mummified, hard, and parchment-like. No organs could be distinguished, therefore to obtain tissue for the experiment the dried-up contents of the upper half of the abdomen and of the chest were scraped out. Bacteriological examination was omitted. In 9 of the 14 cases the bacteria in the blood had been *few* or none, in one "*considerable*," in four "*numerous*." In one of the latter, which had been stored for eighty-five days, a positive reaction ++ was obtained, the remaining cases being negative. The ages of the cadavers ranged from 71 to 100 days, from which must be concluded that putrefaction has not a great, yet a certain influence on the bacterial antigen. In actual experience such mummified cadavers are sometimes sent for examination, but the question has little practical value, since a number of cadavers are sent at the same time, in which case one more suitable may be chosen for experiment (Dunbar and Kister, *l.c.*). In this series the first experience was made of a difficulty mentioned by Ascoli—that of obtaining a clear filtrate from the putrefied tissue. Even after repeated filtration or centrifugalisation, several of the extracts remained opalescent. A slight opalescence did not however obscure a positive reaction, but repeated filtration was a disadvantage when small volumes of fluid were being dealt with, since much is lost by evaporation. Centrifugalisation or filtration through asbestos in a small funnel such as that sold by Messrs L. W. Gans, Frankfort, with the "*Ascoli Anthrax Diagnostikum*," is to be preferred. With such an opalescent solution

a control with extract alone should be carried out in case the extract alone gives a sediment in 16 hours. A clear solution is more readily obtained when the extract is allowed to cool almost completely before filtration.

The reaction in fresh cadavers (up to 48 hours old). Rats 1 to 6 inclusive were injected with $\frac{1}{10}$, 7 to 12 with $\frac{1}{5}$ platinum loop of an agar slope culture. Post-mortem appearances were typical of acute plague, bubo being recognised in all but one case.

Extracts were made from the blood, bubo, spleen, and liver in each case, each organ being first examined as to its bacterial content. In making the films for this purpose, the fresh section of the organ itself was drawn over the coverslip. The results are collected in Table D. The extracts referred to as "*heart-blood*" were made from heart and heart-blood together, the blood alone having been examined in films. The extracts from bubo were invariably opalescent, those from heart-blood occasionally so. Piras states that he obtained clear filtrates by using distilled water instead of normal salt solution as extracting medium. Distilled water was accordingly tried in experiments 4 and 5, the resulting filtrate from the bubo being as before opalescent, and the precipitation result as mentioned below. No bubo was found in experiment 11.

TABLE D.

Experiment	Heart-Blood		Bubo		Spleen		Liver	
	Bacterial Content	Precipitation	Bacterial Content	Precipitation	Bacterial Content	Precipitation	Bacterial Content	Precipitation
1	Few	-	Numerous	++	Few	-	Few	-
2	None	-	"	++	Few	-	Few	-
3	None	-	"	++	Few	-	Few	-
4	Considerable	v. below	"	v. below	Numerous	v. below	Numerous	v. below
5	Few		"		Few		Considerable	
6	None	-	"	-	Few	+	Few	-
7	Few	-	"	++	Considerable	+	Considerable	+
8	Considerable	-	"	++	Numerous	++	Numerous	++
9	None	-	"	++	Few	-	Few	-
10	Few	-	"	+++	Few	-	Few	-
11	Considerable	-			Numerous	++	Numerous	++
12	Few	-	Numerous	++	Considerable	+	Considerable	±

The bubo gave a marked positive result in every case but one, where the negative result must be due to a technical failure, since the bacteria were "*numerous*." The spleen reacted positive in six, the liver in three, of the 10 cases. From an examination of the relative bacterial content of the organs other than bubo, it will be seen that a bacterial content of the spleen and liver large enough to give a positive precipitation result, is only present when septicaemia of more or less degree had been

present at death, a condition by no means always to hand. In Piras' communication no mention is made of this fact, his tables showing constant positive results, although, as seen in the preceding Tables B, C, and D, the degree of septicaemia, even in rats of like size infected with the same quantity of culture, shows all possible variations between the two extremes. Piras further states that he prepared the extracts with distilled water. Now, not only did all the organ extracts from experiments 4 and 5, Table D, which were made with distilled water, react strongly positive, regardless of their bacterial content, but also distilled water itself gave a marked positive result with plague serum, but no trace with normal. This unspecific "*ring*," however, disappeared, leaving no deposit in 24 hours, the period which Piras allowed to elapse before entering his results.

Conclusions. (1) The "Thermoprecipitation" reaction between plague-immune serum and extracts of plague bacilli, whether obtained from pure cultures or from infected organs, is specific ;

(2) and can therefore be used to diagnose plague in cadavers, under the following conditions : the occurrence of a marked positive reaction (+ + +, + +, or +) between undiluted serum and an organ extract is, if the experiment is carried out with suitable controls, viz.

- (a) plague serum + extractive medium (normal salt solution) = negative,
- (b) plague serum + *B. pestis* culture extract = positive,
- (c) normal serum + extract concerned = negative,
- (d) extract, if opalescent, alone = no deposit,

in itself absolute proof that the organ was infected with *B. pestis*. A doubtful reaction (\pm) is merely suspicious of plague, while a negative is of no value, since the organ, though from a plague cadaver, may have contained an inconsiderable number of bacilli.

(3) Because of this last fact, the precipitation can never replace the usual bacteriological methods ;

(4) but by reason of its speed, simplicity, independence of climatic conditions, or putrefaction in the cadaver, and availability in addition to the usual bacteriological methods, it is a valuable supplement to them.

(5) The further application of the method rests on the facts already known as to the distribution of the bacilli in the different organs in the various forms of the disease. Where acute bubo is found—and it is a characteristic appearance also in pulmonary plague and in that contracted by gnawing infected cadavers—the extract would give a positive

result, a negative being definitely against a diagnosis of plague. After the acute bubo, the spleen, and next the liver are the most likely organs to give a positive result. In chronic disease, where, even in the encapsulated local focus, the bacilli are usually scanty, and death takes place from toxæmia, the method would leave the bacteriologist in doubt.

These observations apply particularly to the disease as transmitted by flea-bites, of which infection by subcutaneous injection, as in this research, is the artificial imitation. In the plague cases mentioned by Dunbar and Kister as examined at Hamburg between 1901 and 1903, 59 positive cadavers were sent from four ships. In the case of cadavers from two ships, bubo is expressly mentioned, in all, the macroscopic appearances are described as "typical," suspicious bacilli as "numerous" in the organs. The precipitation method would probably have settled the diagnosis in the four cases within an hour.

In any case, the method recommends itself for trial under actual conditions in a laboratory where the examination of rat cadavers for plague is part of the routine.

NOTE.—The fowl-cholera and swine-fever cultures and sera used in the experiments were gratuitously supplied by the firm of L. W. Gans, Frankfort-on-Main, to whom acknowledgment is made.

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ON SUGGESTED FORMULAE CONNECTING DOSAGE AND DEATH TIME.

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(With Four Charts.)

SEVERAL attempts have been made at various times to obtain a mathematical expression of the relation between dosage, body weight and death time. The subject has recently gained importance through the interesting publications of Dreyer and Walker, who have put forward such a formula, and have illustrated its application to toxicity determinations for a number of substances, and, in particular, for different samples of diphtheria toxin—a matter of considerable practical importance. I propose to examine the theoretical basis of their formula, and its relation to formulae which other workers have suggested, and to criticise the evidence with which they have illustrated its application to experimental material.

The formula proposed by Dreyer and Walker^{1,2} to connect the dose of any toxic substance with the lethal time is

$$\frac{1}{D_0 - a} - \frac{1}{D_1 - a} = k(T_0 - T_1),$$

where D_0 and D_1 are “surface doses” corresponding to the times T_0 and T_1 in which the death of the animal takes place, and a and k are constants for the particular toxin and species of animal used. The surface dose is calculated from the formula $D = \frac{d}{W^{0.72}}$, where d is the actual dose administered, and W the weight of the animal.

¹ *Lancet*, April 11th, 1914, p. 1023.

² *Biochemische Zeitschrift*, 1914, vol. LX. p. 112

The formula consists essentially of two parts, (a) the surface dose, (b) the connexion between dose and lethal time.

(a) *Surface dose.* Dreyer and Walker¹ suggest that the blood volume in warm blooded animals is a function of the body surface. From this they deduce that dosage should be calculated in terms of body surface instead of body weight, on the assumption that the activity of a toxic substance depends upon the concentration in the blood. This assumption may be justified in the case when an acutely acting poison is injected directly into the blood stream. On the other hand, more slowly acting poisons, such as bacterial toxins, are probably fixed rapidly by the tissues, and it is even conceivable that they may have practically disappeared from the blood, before the toxic action is manifest. In such cases the maximum concentration in the blood cannot bear a strict relation to the magnitude of a dose given hypodermically. It is of interest to note that dosage according to surface was recommended some years ago by B. Moore² from quite different theoretical considerations. It is possible, therefore, that Dreyer and Walker's dosage in relation to surface may be correct, although deduced from a doubtful assumption. Within the limits of a single species, and with animals of a sufficiently uniform age and condition to exclude other unknown factors of variation, the correction made by substituting calculated surface for weight will often be within the limits of accuracy of toxicity experiments. The use of $W^{\frac{2}{3}}$ as suggested by Moore and previously by Dreyer and Roy³ will probably yield as accurate results as $W^{0.72}$ determined by Dreyer and Walker, and is much easier to use in dealing with large numbers of experiments. If the doses for two animals are being compared, and the weight of one animal is double that of the other, the difference between the results obtained by the two formulae is less than 4 per cent. If the ratio between the weights of animals is greater than 2 : 1, the comparison between the dosage must be a very rough one, because it is found in practice that there is considerable variation, due to age and condition of the animals, and either formula would give an equally good rough approximation.

(b) *Connexion between dose and lethal time.* Dreyer and Walker, having deduced their equation from theoretical considerations, illustrate its application to a number of data from their own and others'

¹ *Proc. Roy. Soc. B.* vol. LXXXVII. 1914, p. 319.

² *Biochemical Journal*, 1909, vol. iv. p. 323.

³ *Journ. of Path. and Bact.* 1909, vol. XIII. p. 344.

experiments, and claim that it fits the results with great precision. I propose to examine both the theoretical basis of their equation, and their claims to have established it experimentally, the theoretical discussion of the equation being dealt with under the following headings :

1. General criticism of the equation.
2. The theoretical basis of the equation.
3. The value of the constants.
4. Comparison with other suggested equations.

1. *General criticism of the equation.* Dreyer and Walker start from the difficulty of comparing two toxins on the basis of their lethal doses for a standard time, since they find that, with different times, different ratios of activity are obtained for the same toxins. They point out, therefore, that a true measure of toxicity cannot be obtained from the dose that kills in an arbitrarily fixed time. To overcome this difficulty they have suggested the equation

$$\frac{1}{D_0 - a} - \frac{1}{D_1 - a} = k(T_0 - T_1),$$

in which a is the non-effective dose and k a constant which, they state, is the true toxic value of the toxin. Now this statement involves several assumptions.

(a) It is assumed that k and a bear some single relation to one another; otherwise two toxins could exist for which k was the same, but a differed.

(b) It is assumed that if two toxins have the same k and a they kill at the same rate. The equation does not necessarily involve this. It is conceivable that two toxins giving different results may have the same values for the constants; for this to be the case, it is necessary that the lethal time for each dose of one toxin should differ always by a constant amount from the lethal time for the same dose of the other toxin. For instance, for doses D_0, D_1 , etc. the lethal times for one toxin being T_0, T_1 , etc., those of the other toxin might be $T_0 + x, T_1 + x$, etc., and the formula would still hold good in each case; for if it is true that

$$\frac{1}{D_0 - a} - \frac{1}{D_1 - a} = k(T_0 - T_1),$$

it is equally true that

$$\frac{1}{D_0 - a} - \frac{1}{D_1 - a} = k[(T_0 + x) - (T_1 + x)].$$

In other words, the knowledge of the value of the constants a and k can only give the difference between the lethal times for two different doses, and not the actual lethal times.

This point can be made clearer by giving figures for two such toxins. In the following table the lethal times are given for various doses of toxins X and Y . Toxin X corresponds to the theoretical value given by Dreyer and Walker for their toxin C , and toxin Y is an imaginary toxin. In both cases $k = 665 \times 10^{-7}$, $a = 2200$.

TABLE I.

Dose (D)	Toxin X Lethal time (T)	Toxin Y Lethal time (T)
2496	76.0	69.7
2659	57.8	51.5
2788	51.0	44.7
2953	45.0	38.7
3039	43.3	37.0
3283	39.0	32.7
3555	36.3	30.0

The connexion between dose and lethal time for these two toxins is shown graphically on Chart I.

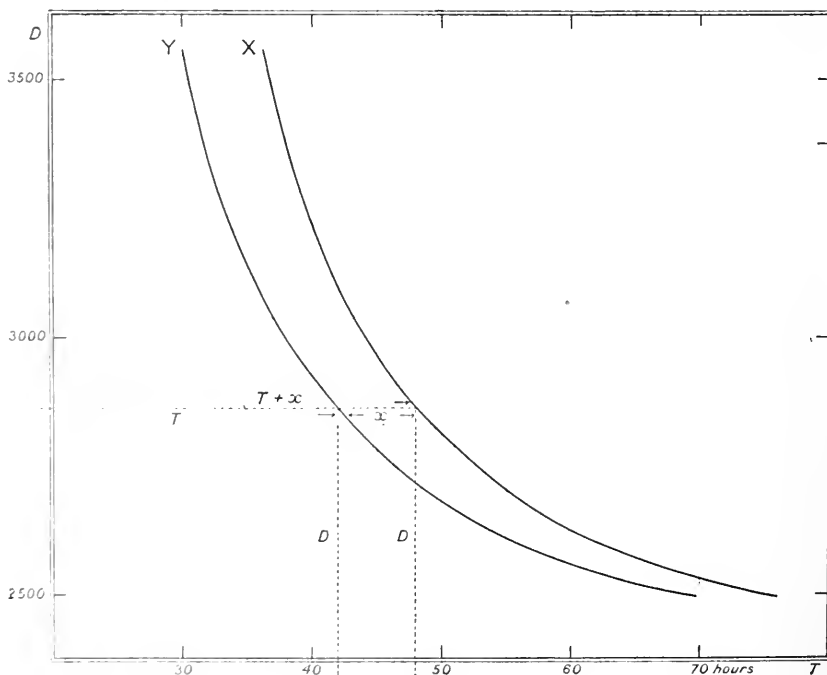


Chart I.

It is, of course, possible that, owing to some inherent characteristic of the action of toxin, no two such toxins as we have supposed can exist, but this has yet to be shown. Dreyer and Walker have applied their formula to diphtheria toxin, and have compared three different toxins by the ratio of the values of the constant k . A consideration of the complex nature of diphtheria toxin, containing, as it does, toxin, toxoids, toxones, etc., leads one to doubt the possibility of a single expression giving a measure of the relative strength of a toxin.

The formula $\frac{1}{D_0 - a} - \frac{1}{D_1 - a} = k(T_0 - T_1)$ cannot fix the strength of any particular toxin, unless, in addition to the constants a and k , the value of T for some value of D is known. A reference to Chart I makes this obvious—if dose be plotted against time the points will lie upon a curve at a constant distance from either of the curves shown on the chart, if the values for a and k are the same. The knowledge of one point upon the chart is necessary before the curve can be drawn representing the connexion between dose and lethal time of the toxin. It follows that, in comparing two different samples of toxin, three values must be known, a , k , and the value of T for a certain value of D . The choice, therefore, of the value of k for comparing the strengths of different samples of toxin would appear an arbitrary one. It might be pointed out here that the necessity of knowing three values for a toxin corresponds with the present method of recording the strength of diphtheria toxin by means of the m.l.d., the L_0 dose and the L_+ dose. It is probably the presence of toxoids and toxones that complicates the comparison of the toxicity of two samples of toxin by means of the ratio of the doses that kill in a stated time. It is possible that in pure poisons, such as inorganic substances, the ratio of the lethal dose remains constant, whatever standard death time be chosen. Dreyer and Walker have shown that this is not the case when the lethal times for male and female *Gammarus* in various concentrations of salt are compared. In this case, however, it must be remembered that the same poison is here compared on two sexes of the same species having different resistance, in place of comparing different toxins on animals of the same sex and species.

2. *The theoretical basis of the equation.* The simplest formula to connect dose with lethal time is $(D \times T) = \text{a constant}$.

This formula as it stands cannot be true, and needs two modifications.

(a) If D is made infinitely large, T must become 0 for this formula

to hold. For no toxin can this be true. In the case of a quickly acting poison (such as hydrocyanic acid or cobra venom), injected intravenously, some short time must elapse between the injection and distribution in the blood stream. In practice, of course, this short time is negligible, but must be taken into consideration when dealing with the subject theoretically. In the case of slowly acting poisons injected subcutaneously, the minimal lethal time is more prolonged, and may reach 12 hours or more for some samples of diphtheria toxin. The value for T , therefore, cannot become 0, but as D is increased so T approaches nearer to the minimal lethal time. It follows that $(T - b)$ must be substituted in the formula for T , where b denotes the minimal lethal time.

(b) If in the formula T is made infinite D would vanish. This again is not true, because, for all toxins, there is a dose that fails to kill. As the lethal time increases, so the dose necessary is decreased, until it reaches the dose that may be termed the theoretical minimal lethal dose, *i.e.* the dose that kills in an infinite time. It is necessary, then, in the formula to subtract this dose from D , in order that this part of the formula may vanish when T is made infinite. It follows that $(D - a)$ must be substituted in the formula in place of D , where a denotes the theoretical minimal lethal dose.

The formula now reads $(D - a)(T - b) = C$. This, as will be shown later, is an alternative expression for the Dreyer-Walker formula.

The connexion between dose and lethal time may not, however, be a simple linear function, but the time may vary inversely as some function of the dose, such as the dose raised to a power or as the logarithm of the dose. In the first case the general formula would read

$$(D - a)^n (T - b) = C.$$

If, however, the lethal time varies as the logarithm of the dose, the constitution of the formula must be slightly altered, so that the logarithm of the expression for the effective dose will be equal to 0 when the lethal time becomes infinite. The formula then reads

$$\log \frac{D}{a} = \frac{K}{(T - b)}.$$

This formula has a reasonable foundation, for if we consider $\frac{D}{a}$ (*i.e.* the number of theoretic minimal lethal doses in the dose injected) to be the stimulus, and the reciprocal of $T - b$ (*i.e.* the delay in lethal time) to be the true measure of the effect, then this formula corresponds with the Weber-Fechner law.

3. *The value of the constants.* The theoretical minimal lethal dose a is the dose that just gives the required effect, *i.e.* causes death; consequently this dose can be considered as the only satisfactory lethal unit. In the case of a pure toxin it seems reasonable to assume that the lethal effect of a dose depends entirely upon the number of lethal units present. We have already seen that the effective dose must be measured as $(D - a)$, and a is the lethal unit, so the lethal effect of any dose D may be expressed as $\frac{D-a}{a}$, where a is the lethal unit or theoretical minimal lethal dose. If we are dealing with a pure toxin whose action is uncomplicated by the presence of toxoids, etc., it appears probable that, apart from variation in rapidity of absorption due to variation in concentration of the solutions injected, equal numbers of lethal units of different samples of toxin would kill in equal times. Since b is the lethal time for an infinite number of lethal units, b must be constant if we can neglect the rate of absorption. It follows therefore that $T - b$ is constant for a given number of lethal units $\left(\frac{D-a}{a}\right)$ of any sample of a pure toxin, so that for pure toxins $\left\{\frac{D-a}{a}\right\} (T - b)$ has a constant value independent of the sample of toxin under investigation. The modified form of the Dreyer-Walker equation reads

$$(D - a) (T - b) = C;$$

it follows, therefore, that $\frac{C}{a}$ has a definite value for each species of pure toxin. Later we show that C in the modified form is the reciprocal of k in the original formula. If, therefore, we accept Dreyer and Walker's equation, $a \times k$ must have a constant value for all samples of one species of pure toxin, provided that any variation in the rate of absorption of different samples of toxin can be ignored, as in the case of intravenous injections.

In subcutaneous injections, the rate of absorption of different samples of toxin probably varies sufficiently to make some slight variation in the values of the constants. When very large doses of toxin are injected subcutaneously, a large number of lethal units must be absorbed very rapidly, and as the dose is increased, so the number of lethal units almost immediately absorbed must be increased, so that theoretically infinite doses, injected subcutaneously, should kill in almost the same time as massive doses injected intravenously, the difference

in time being that which elapses before any of the toxin injected subcutaneously passes into the blood stream. Thus it follows that b , the lethal time for infinitely large doses, is very nearly the same for a toxin whether injected subcutaneously or intravenously. This should yield a simple means for testing whether the suggested values for the constant, calculated for a given toxin, are admissible or not.

Considering the case of more complex toxins, we do not know to what extent toxoids retard or accelerate the action of toxin. If toxoids have any effect then $\frac{c}{a}$ (or $a \times k$) cannot have a constant value, because the same number of lethal units of two toxins, having a different toxin to toxoid ratio, would not kill in the same time.

4. *Comparison with other suggested equations.* Warren's¹ formula for the lethal time of small crustaceans immersed in solutions of various salts is $\frac{1}{T} = k(c - n)$, where k and n are constants, and c the concentration of salt. Warren's formula cannot then be applied to the injection of toxic substances into animals, because, for most toxins, death is not instantaneous when massive doses are injected, and so the formula must be modified to $\frac{1}{T - b} = k(c - n)$, which is the same as

$$(D - a)(T - b) = C.$$

Again Ostwald and Danoscheck's² formula $\frac{1}{T} = k(c - n)^m$ would need modification to $\frac{1}{T - b} = k(c - n)^m$, or using our own notation

$$(D - a)^m(T - b) = C.$$

The formula in this form is a generalisation of all the other suggested formulae. Warren's formula makes $b = 0$, $m = 1$; Ostwald and Danoscheck's $b = 0$; Dreyer and Walker's $m = 1$. Craw and Dean³ have suggested that

$$\text{Lethal Time} \times \sqrt[m]{\text{Lethal Dose}} = \text{Constant, i.e. } D^{\frac{1}{m}}T = C,$$

which corresponds to the general formula with $a = 0$, $b = 0$.

¹ *Journal of Microscopical Science*, 1900, vol. XLV. p. 199.

² *Zeitschrift für Chemie und Industrie Kolloide*, 1910, vol. VI. p. 297.

³ *Journal of Hygiene*, 1907, vol. VII. p. 512.

The connexion between the general formula given above and that of Dreyer and Walker can be shown as follows :

$$\begin{aligned} \text{The formula} \quad & \frac{1}{(D_0 - a)} - \frac{1}{(D_1 - a)} = k(T_0 - T_1) \\ \text{can be rewritten} \quad & \frac{1}{(D_0 - a)} - kT_0 = \frac{1}{(D_1 - a)} - kT_1, \\ \text{or} \quad & \frac{1}{(D - a)} - kT \text{ is a constant.} \end{aligned}$$

Further re-arrangement brings us to the formula

$$(D - a)(T - b) = C,$$

where a has the same value as in the original equation, C is the reciprocal of k , and b is a new constant equal to the minimal lethal time. Expressed in words, Dreyer and Walker's formula states that equal increments in lethal time correspond to equal increments in the reciprocal of the effective dose. The modified form of the same equation states that the product of the effective dose (or increase in dose over the theoretical minimal lethal dose) and the delay in lethal time beyond the minimal lethal time is a constant. Dreyer and Walker were probably fully aware of this modification of the equation, but preferred their own form, although the modification of the equation appears preferable for the following reasons: (1) it can more easily be compared with other suggested formulae, (2) any sample of toxin can be compared with another by means of the values of three constants, instead of two constants together with the value of T for a certain D , (3) in testing the formula upon experimental data the calculations are less involved.

The more general form of Dreyer and Walker's equation to correspond to $(D - a)^m(T - b) = C$ would read

$$\frac{1}{(D_0 - a)^m} - \frac{1}{(D_1 - a)^m} = k(T_0 - T_1),$$

and we have now to consider the experimental evidence given in support of their formula, and whether such evidence is sufficient to warrant the assumption that $m = 1$ if the general formula is correct.

Statistical Evidence.

(a) *Diphtheria toxin.* Tables II, III and IV give the results of Dreyer and Walker's own experiments upon diphtheria toxin. There

is obviously a misprint in their table for toxin A^1 , as the values given for D for each guinea-pig do not agree with the values calculated from the dose and weight in each case. In Table II given below these values have been calculated afresh from $D = \frac{d}{W^{0.72}}$, and the values agree with the average values for D for each group given by the authors.

TABLE II. (From *Biochemische Zeitschrift*, vol. LX. p. 120.)

Number	D	T (in hours)	D (average)	T (average)	T (calculated)
1	1291	220	1291	220	223
2	1315	84	1359	161	161
3	1333	170			
4	1350	192			
5	1443	192			
6	1618	125	1640	83	83
7	1677	45			

TABLE III. (From *Biochemische Zeitschrift*, vol. LX. p. 119; *Lancet*, April, 1914, p. 1023.)

Number	D	T (in hours)	D (average)	T (average)	T (calculated)
1	2370	30	2496	76.0	76.0
2	2552	118			
3	2565	80			
4	2640	100	2659	57.8	58.0
5	2640	38			
6	2661	55			
7	2696	38			
8	2745	64	2788	51.0	50.8
9	2800	38			
10	2820	51			
11	2920	40	2953	45.0	45.2
12	2940	42			
13	3000	53			
14	3015	44			
15	3020	38	3039	43.3	43.1
16	3040	44			
17	3080	47			
18	3220	44	3283	39.0	39.1
19	3230	49			
20	3258	32			
21	3424	31			
22	3525	49	3555	36.3	36.3
23	3560	30			
24	3580	30			

¹ Dr Ainley Walker has kindly pointed out to me that this misprint was corrected in the next number of the *Biochemische Zeitschrift*.

TABLE IV. (From *Biochemische Zeitschrift*, vol. LX. p. 121.)

Number	<i>D</i>	<i>T</i> (in hours)	<i>D</i> (average)	<i>T</i> (average)	<i>T</i> (calculated)
1	1123	180	1268	148	160
2	1311	180			
3	1371	84			
4	1391	60	1415	107	107
5	1404	132			
6	1404	180			
7	1404	96			
8	1427	65			
9	1461	108			
10	1461	72	1514	89	89
11	1461	72			
12	1466	180			
13	1498	62			
14	1591	84			
15	1604	65	1739	67	67
16	1686	64			
17	1781	72			
18	1781	64			

In dealing with the results of inoculation of toxin into animals it must be remembered that individual variation is very great, and little reliance can be placed upon single results.

If we consider the results given in Table II, it will be seen that the number of experiments performed (7) is small, and the agreement between observed and calculated times of death has been based in one case upon only one observation, and in another case upon the average of two observations of animals dying in 45 and 125 hours respectively. From two such discordant results no satisfactory averages can be taken. The other group consists of four observations, one of which (guinea-pig No. 2) dies earlier than four out of five other guinea-pigs injected with larger doses. The three other animals in the same group survived over twice as long as No. 2. The presence of this guinea-pig in the group lowers the average time of death so that the three out of four guinea-pigs in the group die later than the time taken to represent the death time for the group. The averages for the three groups in Table II are plotted below in Chart II, and the actual points from which the averages were taken are marked. It is obvious that, however carefully the scattered points are grouped, the coincidence of their averages upon the curve representing any formula is worthless. If one discrepant point (No. 2) be ignored, the averages for the three groups lie upon a straight line.

Turning to Table III, we find that, where the results are grouped in a certain way, the average of the observed death times for seven

different groups agrees with the calculated death times to within 12 minutes. At first sight this would appear to be strong confirmation of the accuracy of the formula. Considering the table in detail, we find that 76 hours is taken as the average death time for the first group of three observations, with death times of 30, 80, and 118 hours. There can be little reason to suppose that 76 hours represents the true average (within 12 minutes) for that group. Guinea-pig No. 1 can scarcely be included as a true result, as, with a dose represented by the figure 2370, it dies in 30 hours, whereas out of 23 other guinea-pigs, with doses ranging

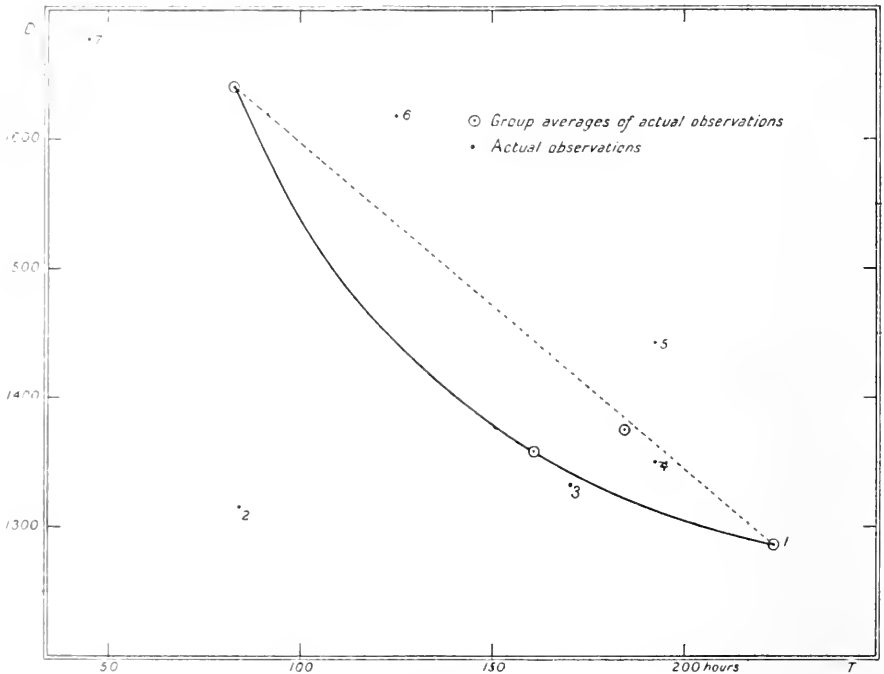


Chart II.

from 2552 to 3580, only two with the largest doses die in so short a time. Again, if we consider No. 22, we find that this animal dies in 49 hours with a dose of 3525; only one other guinea-pig, out of 11 injected with a greater dose than 3000, survives for this length of time. The absence of these two observations would make a very great difference to the average observed death time for the first and last groups, and, as will be seen later, these figures are of special importance.

It is only when a special choice of groups is made that any regularity

of results is obtained. If the results are grouped into divisions so that the extreme value for D does not vary more than 200 (well under 10 per cent.) in each division, we obtain averages as in Table V, which vary considerably from the calculated figures according to Dreyer and Walker's formula, if we accept the value of constants given by them for the particular toxin.

TABLE V.

Numbers 1	Range of value of D	Average value of D	Average value of T	Calculated value for T
	2370	—	30	—
2— 8	2552—2745	2642	70.4	57.6
9—13	2800—3000	2896	44.8	45.2
14—17	3015—3080	3039	43.3	41.5
18—20	3220—3258	3236	41.6	38.1
21—24	3424—3580	3522	35.0	35.0

In Table VI death times are given calculated by substituting various values for a in Dreyer and Walker's formula. It will be seen that with a range of a from 1200 to 2400 and corresponding values for $k \times 10^7$ from 150 to 1126, the calculated death times for the last five groups are very near the observed times, and even for the first two groups they are almost all within the limit of variation. It will be seen that it is only with the lower doses that the value for a makes any appreciable difference to the calculated death times. It is probable that many of the theoretical values of a given in the table could be shown to be inadmissible, if further experiments were made with lower doses. When we consider that a represents the dose that would kill in infinite time, the value for a must be greater than the dose that invariably fails to kill, and allows the animals to recover in weight. If use is made of the modified formula, other values may be discarded as giving an inadmissible value for b which can easily be ascertained by a few experiments upon the lethal time for very large doses.

TABLE VI.

		Calculated lethal times for various values of a						
Group	Average D	Variation in lethal time	Average lethal time observed	$a=1200$	1600	2000	2200	2400
				$k \times 10^7 = 150$ $b = 7.1$	241	450	665	1126
1	2496	30—118	76.0	58.3	60.6	66.4	76.0	121.4
2	2659	38—100	57.8	52.6	53.5	55.3	58.0	63.2
3	2788	38— 64	51.0	48.9	49.2	49.8	50.8	51.8
4	2953	40— 53	45.0	45.0	45.0	45.0	45.2	45.0
5	3039	38— 47	43.3	43.2	43.2	43.1	43.1	42.8
6	3283	31— 49	39.0	39.0	39.0	39.0	39.1	39.0
7	3555	30— 49	36.3	35.3	35.6	36.1	36.3	36.6

The figures given in Table VI show that unless a long range of tests be made no very definite value can be assigned to k which, it is claimed, should be taken as a measure of the strength of a toxin.

On Chart III where values for D are plotted against T the result of each observation is marked together with the average for each group lying on the curve marked D . Considering the distance apart of the various points, any curve, giving points within a few hours of the group averages, must be taken as giving a reasonable formula. Curves B , D , E represent Dreyer and Walker's formula

$$\frac{1}{D_0 - a} - \frac{1}{D_1 - a} = k(T_0 - T_1),$$

with a equal to 1200, 2200 and 2400 respectively (see Table VI). The other two curves A and C represent $(D - a)^m (T - b) = C$ where $m = 2$ and $\frac{1}{2}$, $a = 1200, 2400$; $b = 15$ in each case and $C = 92 \times 10^6$ and 705 respectively. Curve B cannot be considered as giving a true interpretation of the points, but is depicted with the object of showing how important the end points are, for between the doses of 2800 and 3200 all the curves shown coincide within a limit of two hours, a difference which must be taken as negligible, when we consider the individual variation between the separate observations. The chart helps to show graphically how discrepant the results of observations 1 and 22 are from the others. Without these two points the average for the upper group would lie much further to the left, and that of the lower group further to the right. From Chart III it will be seen that curves C and E and possibly A fit the experimental results as satisfactorily as D . We have here a series of 24 observations and find it quite impossible to choose between the formulae

$$\frac{1}{D_0 - 2200} - \frac{1}{D_1 - 2200} = \frac{665(T_0 - T_1)}{10^7},$$

$$\text{i.e. } (D - 2200)(T - 25) = \frac{10^7}{665},$$

$$\frac{1}{D_0 - 2400} - \frac{1}{D_1 - 2400} = \frac{1126(T_0 - T_1)}{10^7},$$

$$\text{i.e. } (D - 2400)(T - 29) = \frac{10^7}{1126},$$

$$\frac{1}{\sqrt{D_0 - 2400}} - \frac{1}{\sqrt{D_1 - 2400}} = \frac{1}{705}(T_0 - T_1),$$

$$\text{i.e. } (D - 2400)^{\frac{1}{2}}(T - 15) = 705.$$

If a number of further observations were made at each end of the scale, so that the curve could be considerably extended towards the asymptotes, the choice of formulae would be more limited. The curves depicted upon the chart by no means exhaust the possible equations, but are merely given for the purpose of showing that, with a very limited portion of a curve, it is possible to apply almost any equation by careful choice of the constants. For example, the logarithmic formula $\log \frac{D}{a} = \frac{k}{T-b}$, if plotted on the chart would be indistinguishable from curve *D* except at the two end points, and even here the difference is very slight.

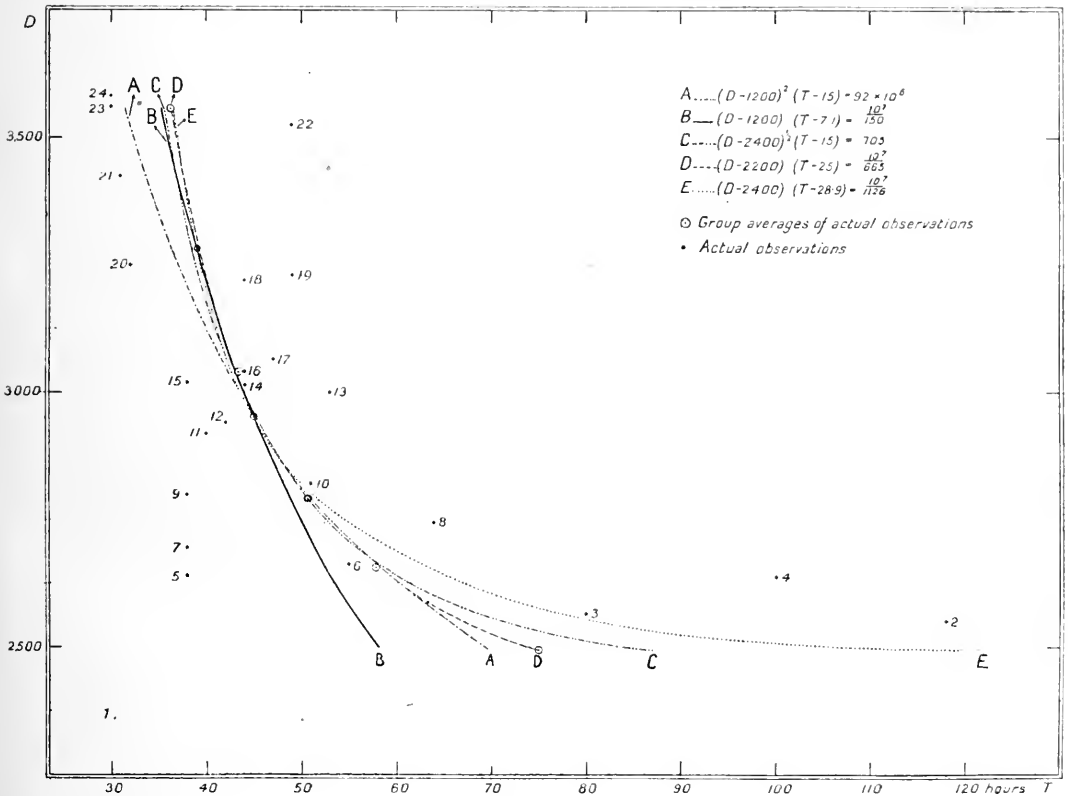


Chart III.

Toxin C (Table IV) again shows careful selection in grouping observations to form averages. It will be seen that three guinea-pigs received the same dose (1461), yet one, dying later than the others, is included in group 2, and the others in group 3. This table also shows the fallacy

of averaging lethal times: in a series of experiments one animal may die considerably later than all the others, and, when a simple average is taken, a much higher figure is obtained than corresponds to the majority of the experiments. In the third group of Table IV five guinea-pigs die between 62 and 84 hours, yet, owing to the presence in the group of one pig (No. 12) with a delayed death, the average lethal time has been taken as 89 hours, which is later than the observed death time for five out of six of the animals in the group, and 18 hours later than the average of the five animals that give consistent results.

Chart IV gives the curves for the following formulae:

$$\begin{aligned}
 \text{(A)} \quad & \frac{1}{D_0 - 1045} - \frac{1}{D_1 - 1045} = \frac{316 (T_0 - T_1)}{10^7} \\
 & \text{or } (D - 1045) (T - 21.5) = \frac{10^7}{316}, \\
 \text{(B)} \quad & \frac{1}{(D_0 - 600)^2} - \frac{1}{(D_1 - 620)^2} = \frac{(T_0 - T_1)}{545 \times 10^5} \\
 & \text{or } (D - 600)^2 (T - 25) = 545 \times 10^5, \\
 \text{(C)} \quad & \frac{1}{\sqrt{D_0 - 1200}} - \frac{1}{\sqrt{D_1 - 1200}} = \frac{(T_0 - T_1)}{1223} \\
 & \text{or } (D - 1200)^{\frac{1}{2}} (T - 20) = 1223.
 \end{aligned}$$

Further observations on the toxin would doubtless show the value of a in curve *B* to be inadmissible. Upon the chart are also marked the individual observations, and it must be agreed that any of the three curves depicted can represent these points equally well. The presence of a number of other points representing the lethal times for much larger and smaller doses would greatly assist in deciding the shape of the curve. The curves marked on the chart are not intended seriously to represent the true results of this toxin, but are arranged to fit in with the average of the groups as arranged by Dreyer and Walker, and it will be seen that, if these averages were truly correct, and obtained from numerous observations, the various curves chosen fit almost equally well. It is obvious that before any formula can be demonstrated to connect dose with lethal time in the case of diphtheria toxin in guinea-pigs, it is essential that a much larger number of observations be made upon each toxin, and that these observations be spread over a much wider range of dosage.

(b) *Other toxic substances.* Dreyer and Walker have also tested their formula upon the results of Schultz for the injection of synthetic adrenalin into mice, and their figures are given in Table VII. It will

be seen again that the coincidence of observed and calculated lethal time depends upon the grouping of the results; observation No. 22 with a dose of 2530 belongs rather to group 5 with doses of 2538, 2663, and 2700 than to group 4 with other doses ranging from 2234 to 2420. This slight, but obviously rational, adjustment alters the average for the groups to D 2323, T 20.6 and D 2608 and T 13.2, bringing

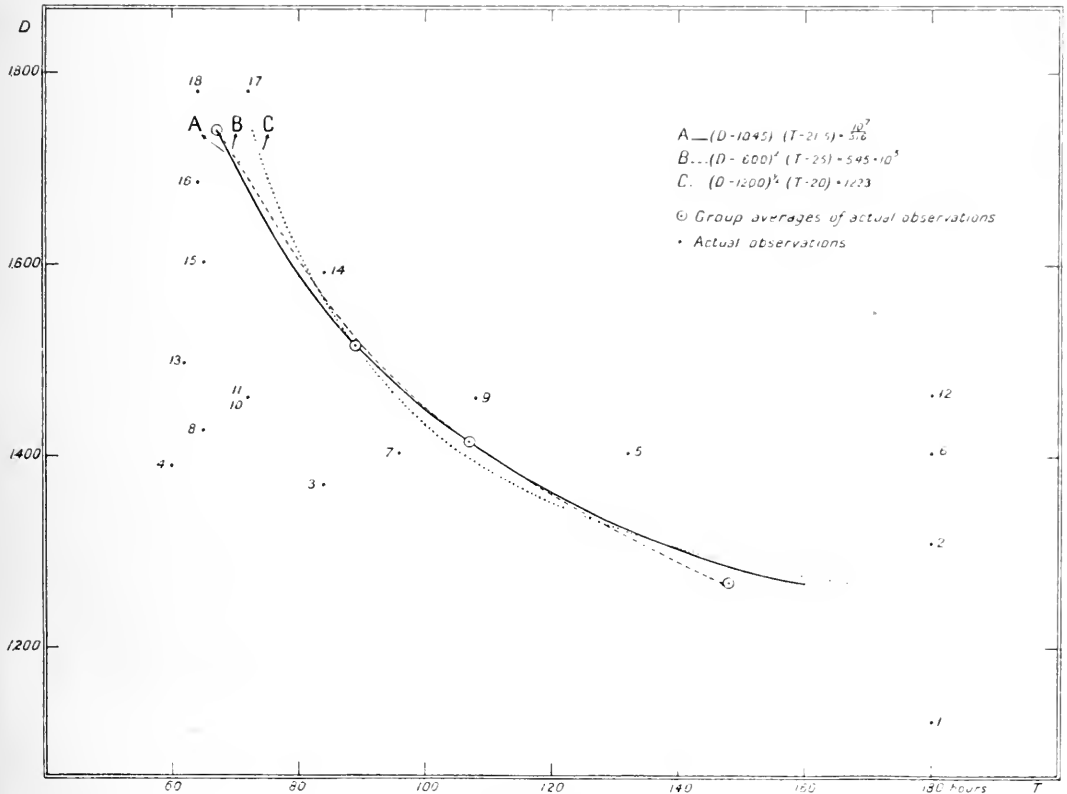


Chart IV.

the observed results still further from the calculated ones. The average for group 1 cannot be considered as reasonable, as it is taken from a group of six observations with a range of death times varying from four minutes to 92 minutes. In addition to this, a reference to Schultz's original paper shows that three mice, that should be included in this group, have been overlooked. These mice (Nos. 170, 258, 262) were all injected within the period of time chosen by Dreyer and Walker with doses

1741, 1622, and 1637, and all lived. We must conclude, therefore, that the result of the second and third groups can only be considered, and it is obvious that there is a great choice of formulae that would fit these figures.

TABLE VII. (*From Biochemische Zeitschrift*, vol. LX. p. 122.)

Number	<i>D</i>	<i>T</i>	Average <i>D</i>	Average <i>T</i>	Calculated <i>T</i>
1	1622	35	1677	30.7	30.7
2	1632	92			
3	1641	11			
4	1699	10			
5	1717	32			
6	1749	4	1886	25.0	25.0
7	1800	17			
8	1803	14			
9	1919	41			
10	1940	33			
11	1970	20	2051	22.0	22.0
12	2002	24			
13	2035	23			
14	2045	22			
15	2073	23			
16	2100	18	2357	18.8	18.2
17	2235	19			
18	2278	27			
19	2320	7			
20	2360	12			
21	2420	38	2634	14.3	15.9
22	2530	10			
23	2538	19			
24	2663	11			
25	2700	13			

Two tables have been published¹ giving the results of individual guinea-pigs injected with cobra venom; these show great variation among themselves and in one case the observed lethal times for six different pigs are compared with the calculated times and the percentage errors are -2 , $+16$, -13 , -24 , $+65$ and -11 per cent.; in the other case eight observations are compared and the percentage errors are -1 , 0 , 0 , -24 , $+66$, -1 , -7 , and $+5$ per cent. The variation is considerable, as would be expected with observations on single animals, and from what we have seen from the curves given above for diphtheria toxin, it is probable that other formulae would fit equally well.

The conclusions drawn from the comparison of Dreyer and Walker's

¹ *Biochemische Zeitschrift*, 1914, vol. LX. p. 124.

formula with that of Ostwald and Danoscheck applied to *Gammarus* are instructive. Two tables are given¹ with 10 observations upon the male *Gammarus* and 11 on the female, and the observed lethal times are compared with those calculated according to the formulae

$$\frac{1}{D_0 - a} - \frac{1}{D_1 - a} = k(T_0 - T_1)$$

and
$$\frac{1}{T} = k(c - n)^m.$$

The difference in the formulae is better seen from the comparison

$$(D - a)(T - b) = C$$

and
$$(D - a)^m \times T = C.$$

Both formulae fit equally well, and the average errors in the two tables are 4.50 and 4.42 minutes for Dreyer and Walker's formula, and 4.48 and 5.94 minutes for Ostwald and Danoscheck's. This shows again how alternative formulae can be applied equally well. In this instance Dreyer and Walker have shown that Ostwald and Danoscheck's figures are inadmissible, in that they have to choose a lower value for the non-effective dose for the more resistant animal than for the less resistant.

A similar objection may be urged against Dreyer and Walker's figures. They state elsewhere in their paper that in "toxic substances of the same quality the weaker toxin has a larger k in the formula." It seems reasonable to suppose that if the formula is applied equally to testing one and the same toxic substances upon unequally resistant strains of a single species, and also to testing different samples of a toxin upon animals of equal resistance, then the toxic substance in the first case behaves to the more resistant strains as a weaker toxin in the second case. It follows that the more resistant male *Gammarus* should give a higher value for k , but we find that the values given by Dreyer and Walker are 841×10^{-6} for the male, and 1055×10^{-6} for the female.

Practical application of the formula. Dreyer and Walker claim that by use of their formula "a great saving both of time and animal material is introduced," and that "the results obtained will also possess a greater validity and a wider application than it has been possible to attain by the use of an arbitrary death time and a fixed standard weight of experimental animals."

¹ *Biochemische Zeitschrift*, 1914, vol. LX. pp. 127 and 128.

It is difficult to see upon what this claim is based. The advantages claimed for the equation may be divided as follows :

- (a) the use of surface doses,
- (b) the connexion between dose and lethal time,
- (c) comparison of toxins by means of their constants.

We will consider these points more particularly in connexion with the testing of diphtheria toxin on guinea-pigs.

(a) *Surface dose.* In the form of $D = \frac{d}{W^{\frac{2}{3}}}$, a formula connecting dose and body weight has been in use for a number of years, but in practice the advantages of using guinea-pigs of a standard weight (from say 240–280 grammes) exceed the disadvantages of selecting animals within a short range of weight. By always using animals of approximately the same weight, the size of local reaction, and the change in weight occurring within two days of the injection, are extremely useful in giving early information of the ultimate result of the test. If the size and weight of animals in constant use is spread over a wide range, interpretation of early results is far more difficult, owing to the variation in standard of measurement. The use of animals of different weights is limited to a range of about 230 grammes to 500 grammes or even less. Outside this range it is found in practice that the individual variation in animals is very great.

(b) *Connexion between dose and lethal time.* It is customary to accept, as the minimal lethal dose of a toxin, that dose that kills say five out of six guinea-pigs within 24 hours on either side of the standard time limit. Very few animals are needed to obtain rough limits within which the required dose must fall, and then about three sets of three, six or more animals (according to the accuracy required), are injected with graduated doses within these limits. To apply Dreyer and Walker's formula, a large number of animals are required to obtain the value of the constants, and the lethal times for at least three doses over a long range must be accurately ascertained. The use of Dreyer and Walker's equation cannot increase the reliability that can be placed upon individual results. In the normal method of testing, isolated results among the orientating tests give rough indications of the dose to be tested ; the actual minimal lethal dose, being tested by direct experiment, prevents any misconception of the strength of the toxin, due to any inaccuracy in such individual results. In the Dreyer and Walker method such individual results may give misleading values for the constants, unless each result is confirmed by a number of others. From

this it appears clear that no saving in animals is effected ; nor is there any saving in time, because, if it is required urgently to know the dose that will kill in a certain time, then, to avoid waiting for the results of orientating experiments, a number of guinea-pigs at each of three or four doses may be injected, but in place of solving the equation for the values of the constants, and again substituting in the equation to obtain the dose for a required lethal time, all that is necessary is to plot the observed times and doses, and obtain the required dose by interpolation upon the curve so obtained.

(c) *Comparison of toxins by means of their constants.* It is difficult to understand any practical need for a theoretical ratio between the toxicity of two samples of toxin. It is usually required either to know the ratio of the dose lethal in a given time, or, more frequently, the comparative immunising values of toxin. In the former case the present method of testing the minimal lethal dose is satisfactory, and, in the latter case, the comparison needed is not between the toxicity of two specimens, but between the binding unit contents. This comparison is made by means of the Lo dose, which can be determined with sufficient accuracy upon far fewer animals than are needed for the determination of the minimal lethal dose.

In Dreyer and Walker's experiments upon three samples of diphtheria toxin, k appears to bear a fairly constant relation to a —for the three toxins A , B and C $\frac{k \times 10^7}{a} = 3.45, 3.308, \text{ and } 3.307$; if this is a true relation for all samples of diphtheria toxin, then the suggestion that the strength of a toxin should be determined by the value of the constant k , resolves into a suggestion of recording the dose that barely kills as a true measure of the toxicity of any sample of toxin, and this appears theoretically to be the true standard to adopt. If, on the other hand, no true relation exists between a and k in complex toxins, then we must again consider the value of each constant a , k and b when recording the value of a toxin, and the basis of comparison made between the toxins must depend upon the result required.

We have already seen, under the heading "The value of the Constants," that in the case of pure toxins injected intravenously it is probable that, if Dreyer and Walker's equation be accepted, $k \times a$ is a constant so that it seems reasonable to conclude that the agreement in the value of the expression $\frac{k}{a}$ for the three samples of diphtheria toxin is purely adventitious.

General Conclusions. Theoretical consideration does not show Dreyer and Walker's equation to be inadmissible.

On the other hand, the experimental evidence published in support of their formula is by no means conclusive, and could be applied equally well in support of other formulae—all suggested formulae and modifications of the general formula

$$(D - a)^m (T - b) = c.$$

Dreyer and Walker's choice of a formula (making $m = 1$ in the general formula) can only be justified if supported by a far wider range of experiments.

I am now testing the formula by data, obtained experimentally, of the lethal times for a wide range of doses of diphtheria toxin.

NOTE ON THE NON-LACTOSE FERMENTERS IN FRESH MILK.

By JOHN RITCHIE, M.B., CH.B., D.P.H.,
Bacteriologist to the County of Dumfriesshire.

IN order to determine whether non-lactose fermenting bacilli are numerous in fresh milk, a series of experiments was made in the early part of this year on samples of fresh dairy milk, taken in Dumfriesshire, for routine bacteriological examination. The samples were brought to the laboratory with the least possible delay, and in no case did more than seven or eight hours elapse between the time of milking and the inoculation of plates. In the majority of instances, the interval was much shorter.

Technique.

(i) The raw milk was plated directly on a series of bile salt neutral red lactose plates—Primary Series.

(ii) Tubes of bile salt neutral red lactose broth were inoculated with 1 c.c. of milk, and plated on bile salt neutral red lactose agar after 48 hours incubation at 37° C.—Secondary Series.

In some cases the sediment from 20 c.c. milk, centrifuged in sterile tubes, was plated out, but in each case with negative results, lactose fermenting colonies only developing.

After incubation, all colourless colonies were subcultured into litmus lactose peptone water, and those which produced neither acid nor gas after three weeks were further studied.

Sixty-one samples of milk were examined, and, though a large number of colourless colonies were isolated, only seven samples (11·47%) proved to contain non-lactose fermenters. From these seven samples, eight organisms were obtained. These were put through the tests devised by Graham-Smith, Lewis and Moore Alexander¹, with the results shown in Table I.

¹ *Report of the Medical Officer to the Local Government Board, 1911-12.*

TABLE I.

Number	Glucose	Mannit	Dulc	Saccha- rose	Salicin	Sorbit	Indol	Motility	Milk	Gelatine	Type
M 1	AG	AG	-	-	-	-		+	A then Alk.	-	Ha 10
M 2	-	-	-	-	-	-	+	-	-	-	A 3
M 3	-	-	-	-	-	-	-	-	-	-	A 1
M 4	AG	AG	-	-	-	-	+	+	A then Alk.	-	Ha 12
M 4 (a)	AG	AG	-	-	-	-	-	+	A then Alk.	-	Ha 10
M 5	-	-	-	-	-	-	-	+	-	-	A 2
M 6	A	-	-	-	-	-	-	-	AC	-	Ba 17
M 7	AG	AG	-	AG	AG	-	-	+	-	-	Hc 2

Thus of the seven positive samples, three contained organisms of Group A, three of Group H, and one of Group B.

In regard to the general bacteriological condition of these samples, lactose fermenters were absent from 1 c.c. in one case, present in 1 c.c. in two cases, in 0.1 c.c. in three cases, and in 0.001 c.c. in one case.

Although it is probable that a more satisfactory technique might show a higher percentage of positive cases, these results suggest that non-lactose fermenters do not occur in large numbers in fresh milk.

THE PHOSPHORIC OXIDE CONTENT OF MAIZE FLOUR.

BY J. McCRAE, PH.D., F.I.C.,
Government Analyst, Transvaal.

(From the Government Chemical Laboratories, Johannesburg, Transvaal.)

IN 1909, in a paper published from the Federated Malay States Institute for Medical Research, Fraser and Stanton in discussing the etiology of beri-beri found that polyneuritis could be produced in fowls by a diet of polished white rice and that the fowls remained healthy if, in addition to the polished rice, they received a sufficient quantity of rice polishings or a quantity of an alcoholic extract (evaporated at a temperature of 52° C.) of whole rice. They established the fact that the essential cause of beri-beri was to be sought for in a nutritive defect, and they endeavoured to determine by chemical methods precise differences between rices capable of causing polyneuritis in fowls and rices which did not cause the disease. Their experiments had shown conclusively that highly polished rice gave rise to the disease but whole rice did not and that the disease could be cured by adding rice polishings to the diet. The chemical examination showed that, whereas whole rice contains about 0.469 per cent. of phosphoric oxide, polished white rice contains only about 0.277 per cent. and the polishings contain about 4.2 per cent.

At that time Fraser and Stanton did not claim that the disease was due to a deficit of phosphoric oxide in the diet, but they claimed that the amount of phosphoric oxide in a rice could be used as an indicator of the disease-producing power of the rice: the lower the amount of phosphoric oxide in a polished rice the more likely is it to cause polyneuritis in fowls or beri-beri in man.

Later work by Fraser and Stanton showed that phosphoric oxide or phosphorus compound is not the substance in whole rice or rice

polishings which prevents the disease, and researches by other workers have amply confirmed this.

Several investigators (Fraser and Stanton, Funk, Schaumann, Eadie and Simpson, etc.) have contributed towards the solution of the problem as to the nature of what may be termed the preventive substance occurring in grain. The subject has been completely reviewed by Casimir Funk in his book on *Die Vitamine* (Wiesbaden, 1914). It has now been proved that vitamine is the preventive substance and that it occurs in greatest abundance in the germinal portion and in the outer layers of the pericarp of the grain. The constitution of vitamine has not yet been established, but it is certain that vitamins from different grains and other natural products (yeast, meat, milk, etc.) are not identical.

The isolation of vitamine from a foodstuff is a tedious process and is not available as an analytic method. Fraser and Stanton have suggested that the amount of phosphoric oxide in rice may be used as an indicator: although vitamine contains no phosphorus, yet since the portion of the grain in which the vitamine is concentrated is also the portion richest in phosphorus, Fraser and Stanton's suggestion is probably well founded when applied to grain or grain-products which have been subjected only to mechanical processes and not to extraction processes and which have not been heated to too high a temperature (which is detrimental to vitamine). For, if that portion of the grain rich in vitamine is lost in the milling process then the waste must also be richer in phosphorus and the milled flour must be correspondingly poorer in phosphorus. Much experimentation has established that this is the case with rice, and in the Philippines "a rice containing less than 0.4 per cent. of phosphoric oxide is regarded as polished and that which contains a greater percentage of phosphoric oxide as unpolished rice" (Victor G. Heiser, *Philippine Journ. Sci.*, B. Med. Sci., 1911, vi. 229).

If this holds for rice it seems highly probable that it applies to other grains also. If in the grinding of a grain the flour produced is poorer in phosphorus than the original grain, then it is almost certain that some of the vitamine present in the grain has been lost in the milling. Since vitamine occurs in small proportion in foodstuffs, it is advisable to avoid the loss of any of it if possible.

In order to ascertain if the loss of vitamine (as measured by the loss of phosphoric oxide) in the usual production of maize meal or flour is serious a number of samples of maize and of the flours and

wastes therefrom were obtained from mills on the Witwatersrand, Transvaal.

The milling practice varies considerably, and in most cases a fine white flour of good appearance is produced: this flour represents, as a rule, from 84 to 89 per cent. of the original maize. From 6 to 9 per cent. of bran is produced and about 3 to 5 per cent. of seconds: 1 to 3 per cent. of waste (including dirt, stones, wire, nails, string, etc.) is removed at the mill.

In the samples examined the water and ash were determined as well as the phosphoric oxide. In estimating the phosphoric oxide the precautions advocated by Leavitt and LeClerc (*Journ. Amer. Chem. Soc.*, 1908, xxx, 391, 617) were observed.

It is needless to record here all the results obtained: the following three examples are typical, and in *every* case it was found that the fine meal (flour) contained not more than four-fifths of the phosphoric oxide present in the original grain.

	% Water	% Ash	% Phosphoric oxide
A. Whole maize	11.5	1.11	0.55
Fine meal	10.9	0.94	0.44
Seconds	10.8	3.64	0.78
Bran	10.2	2.10	0.70
Waste	10.9	4.20	0.70
B. Whole maize	12.1	1.01	0.45
Fine meal	10.7	0.72	0.32
Bran	10.3	2.21	1.08
Waste	9.7	2.19	0.81
C. Whole maize	11.5	1.18	0.53
Fine meal	11.6	0.86	0.40
Waste No. 1	9.7	13.03	0.62
Waste No. 2	10.3	8.73	1.05
Waste No. 3	10.5	16.05	0.54
Bran	10.2	3.38	1.23

Incidentally it may be mentioned that samples of "samp" (consisting only of maize endosperm) contained only from 0.16 to 0.20 per cent. of phosphoric oxide.

Many analyses proved that in the conversion of the grain into flour a loss of phosphoric oxide always took place and it is presumed that concomitantly there was a loss of vitamine. The loss is not so considerable as takes place in the polishing of rice, and it is probable therefore that a diet of this maize meal does not induce such serious effects as a diet of polished rice does, but as maize meal is a staple

article in the diet of the native labourers at the Witwatersrand gold mines it seemed desirable to ascertain if, by milling the grain in such a way as to minimise the loss of phosphoric oxide (and vitamine), the flour would be improved. For experimental purposes the milling practice at a mill was altered in such a way that 96 per cent. of the original grain was converted into fine meal passing a 30-mesh sieve. Many analyses of the product have been made, and it has been proved that the fine meal contains nearly as much phosphoric oxide as the original grain. The following analyses are typical :

		% Phosphoric oxide				
		A	B	C	D	E
Whole maize	..	0.53	0.49	0.48	0.57	0.45
Fine meal	..	0.51	0.46	0.46	0.53	0.45

The loss of phosphoric oxide (and with it the loss of vitamine) has been reduced to a minimum and dietary experiments have proved that very considerable advantage has thereby been achieved.

Experiments on a large scale with about 6000 native labourers are being conducted by Dr D. Macaulay with the old type of meal and with the new. Half of the labourers receive in their rations the old type and the others receive the new. Dr Macaulay will report later on his experiments, but I have his permission to state that the incidence of disease amongst those using the new type of meal is much lower than that amongst those using the old type. Further, no intestinal disturbances due to the use of the new type of meal have been observed. The conclusion appears to be justified that fine white meal, produced after removal of the husk and a considerable proportion of the germinal portion of maize, is a defective foodstuff which may give rise to some form of deficiency disease: by grinding the maize in such a way that practically the whole of the grain is converted into fine meal this defect is remedied.

STUDIES ON THE EFFECT EXERTED BY SHAKING ON SERUM.

By HANS SCHMIDT

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INTRODUCTION.

OWING to the difficulty of satisfactorily explaining many facts in serological work, there has been a tendency in the last few years to consider serological phenomena more and more from a physico-chemical point of view.

A serum, physically considered, is a protein hydrosol containing electrolytes. Different proteins, globulins and albumins, are kept in the serum in the relatively stable state of an hydrophile colloidal solution both by their mutual protecting influence as well as by the influence of the salts. Under the most diverse conditions of a purely physical nature, such as heat, storage, freezing, decrease of salt concentration by dilution, or loss of salts by dialysis, increase of the surface by shaking, etc., the stability of the colloidal phases is altered and more or less energetic alterations of the physical state of the serum take place. These may be reversible or irreversible and are followed by changes in the serum as regards their serological properties.

Shaking a serum produces well-marked alterations in it, visible to the naked eye first of all as cloudiness and followed later by precipitation. The production of cloudiness in a serum by shaking has been already observed by Jakoby and Schuetze (1910), but not until recently in the work of P. Schmidt and M. Liebers (1913), H. Schmidt (1913) and L. Hirschfeld and R. Klinger (1914) has this phenomenon been especially taken into account and recognised as the main alteration which a serum undergoes during shaking and of which all other changes observed are more or less a consequence. Seeing the increase of surface to be the main effect of shaking, and that the mechanical agitation by itself plays

a less important part, probably only in continuously renewing the surface, it may be assumed that the alteration in the serum as the effect of shaking occurs more quickly the greater, *ceteris paribus*, the surface is, to which the serum is exposed. It is therefore evident that the intensity of the shaking influences greatly the time necessary to produce cloudiness in a serum, for the more intensely shaken a serum is, the finer is the froth and the greater is the development of the surface. S. and S. Schmidt-Nielsen (1909), in their experimental work on the inactivation of rennet by shaking, have observed to what extent the difference in the ratio of shaken liquid to bottle volume produces a variation of the results. Ritz (1912) also drew attention to this relation as regards shaking of serum and in a former paper (1913) I fully confirmed this.

In order to obtain comparable results, the ratio of bottle volume and liquid as well as the intensity of the shaking movement must be kept constant.

Factors concerned in the effect of shaking upon serum.

Supposing that these relations are kept throughout under optimal conditions, there are still many other factors which may by their presence either accelerate or inhibit the effect of shaking on serum. Before quoting them it should be mentioned that all the following observations are made on fresh guinea-pig serum. The complementing power of such a serum is a characteristic property of its freshness and will become lost by any factor which may alter the physical conditions of the serum proteins. The influence which shaking especially exerts upon the complementing power of a serum will be dealt with in a further communication. It is proposed here merely to deal with the effect of shaking in producing coagulation and denaturation of the proteins of a serum.

The concentration of the serum.

In the first experiments made to inactivate the complement of a serum by shaking, it was found by Jakoby and Schuetze (1910) that a longer and more intense shaking is required to render a serum inactive when undiluted, and therefore these authors used the usual dilution of 1 : 10 in their experiments. This dilution has been later found by Ritz (1912) to be an optimum, and my results, published in a former paper (1913), partially confirmed this. In the meantime I found, however, that frequently a serum diluted 1 : 20 or even 1 : 40 with 0.85 % saline

showed the same degree of cloudiness and inactivity as regards complement action as when diluted 1 : 10. If the inactivation by shaking is due to change in surface energy, I can see no reason for an optimal dilution. The process is to be considered as progressing with time. The shaking causes aggregation and coagulation of a certain part of the proteins, as will be more fully explained later. This coagulation affects only a part of the total proteins. Now, since the relation between volume of liquid and gas as well as the shaking movement are kept constant, thus producing by the equal intensity of shaking an approximately uniform froth and therefore an approximately similar extent of surface, the relation of the denaturated protein to the total protein-content will be the smallest in the undiluted serum, which therefore needs a greater time to become altered to the same extent as in the diluted serum. The relation of the amount of the unchanged proteins to the denaturated proteins increases with the concentration of the serum, as Schmidt-Nielsen also found in the inactivation of rennet by shaking. If the complementing power of the serum be taken as a measurement of the effect produced by shaking, the decrease of complementing power by shaking will be partially covered by that produced by dilution. In the case of a serum the conditions are more complicated than in the case of ferments as rennet or pepsin, etc., inasmuch as the shaking produces principally a denaturation of the euglobulins, as well as an alteration of the albumins, which latter play an important part in the stability of the globulins. Since dilution of the serum decreases the absolute content of albumins, this factor must be taken into account, when considering the more rapid inactivation of diluted sera effected by shaking.

Concentration of salts in the serum.

If instead of 0.85 % saline, dist. water is taken as dilution medium, or if NaCl crystals are added to the native serum, the concentration of salts will be altered and, apart from the effect of shaking, this alone will account for a change in the stability of the colloidal proteins.

a. Decrease of salt concentration.

Sachs and Teruuchi (1907) found that dilution 1 : 10 with aqu. dist. and standing $1\frac{1}{4}$ hours at 37° C. will affect a complement-containing serum in such a manner as to render it inactive, if it is again made isotonic. They found that this phenomenon took place more rapidly










at higher temperatures and that a dilution of 1 : 10 was an optimum. Such a serum could be reactivated by addition of the albumin fraction obtained by dilution and acidification with CO_2 , while the euglobulin fraction was rarely able to do so, and only then to a lesser degree [Sachs and Bolkowska, 1910]. Marks (1912) found that a serum after dilution to 1 : 10 with aqu. dist. and standing $1\frac{1}{4}$ hours at 37°C . was not completely inactive. The fractions of a serum so treated obtained by CO_2 gave full action, when combined with the corresponding fractions of a normal serum. Bessemans (1913) extended these observations and found that the fractions were inactive when combined with themselves. If the fractions of a normal serum were diluted 1 : 10 with dist. water and then kept for $1\frac{1}{4}$ hours at 37°C ., he found, generally speaking, no loss of power in their action. Sometimes a slight reduction could be observed in the action of the globulin fraction, as also observed by Guggenheimer (1911), but no alteration of the albumin fraction ever occurred. Diluted with aqu. dist. produces decrease of the salt concentration. This alters the conditions controlling the solubility of the euglobulin, part of which is therefore thrown out of solution in the form of a suspension, which renders the serum opalescent and cloudy. Addition of salt brings the suspended euglobulin promptly back in solution. After standing a long time however the suspended and precipitated euglobulins undergo alterations, becoming insoluble. The solubility of the euglobulins is favoured by the presence of serum albumins. A solution of euglobulin obtained only by means of salt is always more or less opalescent, indicating that there is no true solution. According to H. Chick (1913) electrically neutral compounds of the salts with the euglobulin are formed, which Schryver (1910) supposes to be due to adsorption. In the serum the albumins, owing to their lower surface tension value, exert a protecting influence on the euglobulin in solution, causing a greater dispersity and thus a true solution of the latter. A more detailed reference to these relations will be given later.

In view of the importance of the relationship between the effect of dilution and standing and the effect produced by shaking, I repeated the experiments of Sachs and Ternuchi and give below the results obtained.

Technique :

Haemolysis was due to the complementing action of the serum in combination with sensitized sheep red corpuscles, of which a 2.5 % emulsion in 0.85 % saline was used. Sensitization was effected with three times the single lysing dose of an inactivated rabbit immune serum.






























The following schema has been adopted to illustrate different degrees of haemolysis :

-  No haemolysis.
-  Faint trace of haemolysis.
-  Very slight haemolysis.
-  Well-marked haemolysis.
-  Half haemolysed.
-  Strong haemolysis.
-  Very strong haemolysis.
-  Nearly complete haemolysis [a slight trace of unlaked cells at the bottom].
-  Complete haemolysis.

*Experiment showing the influence of dilution with dist. water
upon a complement-containing guinea-pig serum.*

Fresh guinea-pig serum was diluted 1 : 10 with aqu. dist. and kept for $1\frac{1}{2}$ hours at room temperature and at 37°C . Then the serum, rendered isotonic, was tested for its haemolytic power with 0.5 c.c. sensit. red cell emulsion (AB dose = 3 times the single lysing dose).
















Haemolysis after 1 hour at 37° and 15 hours at room temperature.
Total volume in each tube 2.0 c.c.

				1.0	0.5	0.25	0.15	0.0
C_n = normal serum dil. $\frac{1}{10}$ with NaCl	...							
C_r Dil. $\frac{1}{10}$ with aqu. dist. $1\frac{1}{2}$ hours at room temp. then rendered isotonic	C_r						
	+ 0.5 c.c. E_n							
	+ 0.5 c.c. M_n							
C_t Dil. $\frac{1}{10}$ with aqu. dist. $1\frac{1}{2}$ hours at 37° , then rendered isotonic	C_t						
	+ 0.5 c.c. E_n							
	+ 0.5 c.c. M_n							

The thus treated sera, before being rendered isotonic, were treated by CO_2 according to Liefmann's method. The globulin fraction, = M-piece, was not taken up in solution by 0.85 % NaCl until immediately before use. The albumin fraction, = E-piece, was filtered through hardened paper and then rendered isotonic.

The fractions thus obtained from C_n , C_r , C_t are respectively E_n , E_r ,

E_t , M_n , M_r , M_t . 1 c.c. of each gave with 1 c.c. of each other and 0.5 c.c. of the sensit. red cell emulsion, the following reactions :

E_n + saline		E_n + M_t	
M_n + ..		E_n + M_r	
E_n + M_n		E_r + M_n	
E_r + saline		E_r + M_t	
M_r + ..		E_t + M_n	
E_r + M_r		E_t + M_r	
E_t + saline			
M_t + ..			
E_t + M_t			

The experiment shows that inactivation has taken place, but more completely in the case of the serum which has stood at 37° C. But contrary to the statement of Teruuchi, I found that a fresh globulin fraction of a normal serum exerted a better restitution effect than the albumin fraction.

As regards the CO_2 -fraction of the serum, treated by dilution with aqu. dist. and standing, it follows from the above experiment that the alteration concerns only the globulin fraction, the albumin fraction of a serum thus treated proving to be active when combined with a normal globulin fraction.

If the serum however was not fresh, I found that dilution with dist. water and standing had a far less destructive effect upon the complementing power of a serum. Inactivation will not be complete, even after many hours standing. Cf. Exp. 6 in my paper on the effect of Berkefeld filtration upon Complement (this *Journal*, 1914). What process has taken place during dilution and standing so as to render the complementing power of a serum inactive is difficult to say, for the suspended globulins seem to be in no way altered, the serum becoming quite clear if rendered isotonic. Probably some alteration in the H⁺-concentration takes place, which may inhibit complementing action, but no definite evidence can yet be given in favour of this conception.

Now, if such a serum, the euglobulins of which are in a state of lability owing to lack of sufficient salt concentration, be shaken, it is evident that these euglobulins will aggregate and coagulate more readily.

In fact, a serum diluted 1 : 10 with aqu. dist. is very rapidly altered by shaking, so that the cloudiness, which in the unshaken control serum will promptly vanish by addition of salt, will not disappear if the serum be rendered isotonic, but will do so on the addition of alkali, thus indicating that the protein particles have undergone denaturation.

Increase of salt concentration.

An increase in the concentration of salt has a somewhat opposite effect upon the influence of shaking. Since Friedberger (1908) drew attention to the use of concentrated salt solution for the preservation of the complementing power of a serum, this method has been frequently and successfully used [Hecht, 1910]. As already mentioned, a certain concentration of salt is necessary for the stability of the euglobulin in the serum [H. Chick (1913)]. This protection however is only a relative one, as the euglobulin is also precipitated in the course of time in a native and otherwise untreated serum. Higher salt concentration prolongs the time during which a serum can be kept clear, by maintaining the dispersity of the protein-phases and thus preserving the complementing power.

If a serum is shaken in a hypertonic condition the time necessary to cause a denaturation and coagulation of proteins will be found to be prolonged, but even as high a concentration as 20 % will not absolutely protect the serum, as well as the isolated protein solutions, from becoming denaturated, as will be seen later. The protecting influence of salts upon the effect of shaking is therefore relative, which fact has already been recognised by Hirschfeld and Klinger (1914) to be the case in human serum.

The influence of temperature upon the effect of shaking.

The accelerating influence of a temperature of 37° C. upon the effect of shaking has been observed by all authors who have worked on the shaking inactivation of complement and ferments. The denaturation and precipitation of a part of the serum proteins occur also at low temperatures. Seeing that it is principally a surface phenomenon, this would be expected to be the case. The acceleration observed at 37° C. is a summation of the effects produced by the temperature and that produced by the shaking. An explanation of this very complicated process will be attempted later. Here, only the fact may be mentioned.

If, however, on the other hand, a serum has been previously heated, the time required to produce cloudiness and coagulation by shaking will be found to be prolonged, the more so the greater the temperature to which the serum was heated. This observation has only been made by Hirschfeld and Klinger (1914) in the case of human serum. They found that exposure to a temperature of 58°C . for 1 hour prevented the serum from becoming cloudy on shaking, as well as by dilution with aqu. dist. They postulate that thermoinactivation as well as a hypertonic condition, the latter in a lesser degree, renders the serum euglobulins more stable: an explanation of this fact however was not given. I think a probable explanation can be given by the results of observations made by H. Chick and C. J. Martin (1913) and H. Chick (1913) in the following way.

The denaturation process of proteins as the result of high temperatures has an abnormally high coefficient of temperature, which apparently effects the denaturation of proteins and their coagulation in the serum to be due to a certain temperature. In reality the process of denaturation takes place already at lower temperatures, but proceeds very slowly. It can however be observed to take place at 37°C . under favourable conditions. The euglobulins form salt compounds in the serum, which remain electrically neutral, when enough salt is present to prevent their dissociation. If the euglobulin has been denaturated by heat, and there is evidence that an exposure to 56°C . for half an hour is sufficient to produce heat-denaturation, the euglobulins lose their characteristic property of forming electrically neutral solutions with salts. The euglobulin particles become electrically charged and any change in dispersity is prevented by their mutual repulsion. The alkalinity of the serum is increased. A decrease of H^{+} -concentration takes place [S. T. Sørensen and Jürgensen (1911)], and the dispersed protein particles are negatively charged. As long as in such a serum the H^{+} -concentration is kept constant, the stability of the euglobulins remains increased.

This may probably be the principal reason why sera previously heated remain clear much longer on standing and require a considerably longer time to become visibly affected by shaking.

I have often observed in the case of guinea-pig serum that half an hour's heating at 56°C . does not prolong very much the time necessary for coagulation by shaking, but a longer exposure to 56°C . prolongs considerably this time, and I was unable to bring about a visible coagulation by shaking in the opalescent serum diluted 1 : 10 with saline and exposed to the temperature of boiling water. It may be possible that

the resistance against the shaking effect, which can be observed in heated sera, may be partly due to the influence of capillary-active substances, which are formed in the serum during heating and which lower the surface tension against air. Further reference to this will be made later.

Influence of the nature of the gas.

That the coagulation of proteins by shaking is not dependent on the nature of the gas, which is in contact with the serum surface, in so far as it is chemically inactive, has already been shown in the early experiments of Melsens (1851), Harting (1851) and Smee (1863, 1864). Metcalf (1905) stated that the formation of membranes of the surface of colloidal solutions was independent of the oxygen and Ramsden (1894) found that all solutions, the particles of which coagulate under the influence of shaking, show this phenomenon also, if oxygen is excluded. Similar results were obtained by A. O. Shaklee and Meltzer (1909), who found that the inactivation of pepsin by shaking took place equally well whether the experiment was done in O, H, or in CO₂. Contrary to this, Courmont and Dufourt (1912) concluded from their experiments that the inactivation of the complement in a guinea-pig serum by shaking was due to oxidation. This could not be confirmed by P. Schmidt and M. Liebers (1913) nor by myself (1913) working independently. No evidence was found for oxidation being responsible for the shaking effect. However, instead of shaking the serum in oxygen, as I formerly did, in order to determine what rôle, if any, oxidation plays in shaking inactivation, I exposed the complement-containing serum to the influence of oxygen in nascent state obtained by the action of the serum katalase on hydrogen peroxide. I found that if H₂O₂ was added to the serum in such quantities as to produce an 0.5 % H₂O₂-solution, no evidence of loss of complement could be observed in spite of the large amount of oxygen thus developed in the serum, enough being present to render the serum colourless in a few minutes. I am therefore convinced that the effect of shaking on serum is not due to oxidation.

Influence of the age of the serum upon the shaking effect.

Storage of a serum diminishes its complementing power. This loss runs parallel with the formation of a cloudiness and of a precipitate. Apart from the influence of bacterial growth, which according to Hara (1913) accelerates the loss of complementing power, this decrease of complement and the formation of precipitates occur also under sterile

conditions. The precipitates in old sera consist of euglobulin, which has become more and more insoluble. It has been seen that hypertonic condition as well as previous heating increase the stability of the euglobulin to this extent, that sera so treated remain clear for a considerably greater length of time. If a serum which has been kept for a long time be treated by CO_2 according to Liefmann's method, I found that the action of the fractions, thus obtained, varied according to the length of time the serum has been kept. Great individual variations however occur. Generally speaking I found, at the commencement, when the complement activity was not yet completely lost, that both fractions gave a complete action when combined with the corresponding fractions of a fresh serum. Later on the globulin fraction of the old serum loses its power to reactivate a fresh albumin fraction, but not *vice versa*. Finally the albumin fraction of the stored serum loses its action. This however was not found to be of constant occurrence, for sometimes a serum was found, the globulin fraction of which retained its activity with a fresh albumin fraction as long as the albumin fraction did with a fresh globulin fraction. This however must be considered as exceptional. Changes taking place in the euglobulins in the course of storage have been already noted by Liefmann (1911) and Mutermilch (1911). They found that in sera kept for a long time the albumin fraction was the more stable of the two, and this has been confirmed by Bessemans (1913) more recently.

A probable explanation of the process is, that first of all an adsorption of the albumins in the free surface takes place. This size of the surface is of importance, for I could observe that with an increase of the surface against air there is an increase in the rapidity with which the complement of a serum is destroyed. This adsorption of albumins on the surface diminishes the stability of the euglobulins, leading to a gradual precipitation of the latter. At the same time adsorption of salts is possibly taking place, for according to Hecht (1914) the electrical conductivity decreases with the length of time.

The process of inactivation due to storage then closely resembles that produced by shaking. The same alterations occur, but more rapidly, and are therefore followed by a much more rapid loss in the inactivity of the albumin fraction.

It is interesting to notice that the greater stability of the euglobulins produced by heating is paralleled by the observation of Mutermilch (1911), who found that under similar conditions the time required to produce precipitation of the euglobulin by dialysis is considerably

prolonged. The same but to a lesser degree has been found by him (1911) to be the case in an old serum.

This is precisely what occurs with regard to the production of a precipitate in a serum by means of shaking, a well-marked inhibition occurring in the case of a heated serum and a relatively lesser inhibition in an old serum.

The effect of shaking upon the isolated proteins of a serum.

In regard to all these factors, which either accelerate or tend to inhibit the effect of shaking, it is highly probable, that the shaking of a serum results in rendering the euglobulins unstable and thus increasing their tendency to aggregate and precipitate. That the effect of shaking is really of such a nature is rendered certain by a study of the effect of shaking upon the isolated proteins. I undertook first of all experiments showing the effect of shaking upon the isolated fractions, obtained by Liefmann's method [dilution with dist. water and acidification with CO_2]. The precipitate which, re-dissolved in 0.85 % NaCl, represents the globulin fraction, contains the euglobulin and a relatively small part of the pseudoglobulin, which can be demonstrated by the behaviour of the solution, when 1 : 3 or 1 : 2 is saturated with the sulphate of ammonium.

The same method of salting out reveals the fact that the albumin fraction of the serum, *i.e.* the supernatant fluid rendered isotonic, contains all the albumin and the main bulk of the pseudoglobulin.

Now, if the albumin and globulin fractions are shaken under similar conditions the following results are obtained.

The shaking of the globulin fraction produces relatively little and unstable froth, and in a very short time cloudiness is developed and precipitation takes place. If the precipitate is removed by centrifugation, and the shaking continues, no more cloudiness will appear, the liquid remaining clear. Half saturation with the sulphate of ammonium reveals the presence of some pseudoglobulin, euglobulin no longer being present. If the salt concentration of the globulin fraction is increased to 20 %, the time required to coagulate the euglobulins is found to be prolonged but coagulation finally does take place.

The shaking of the albumin fraction however is accompanied by the production of a copious and persistent froth and the liquid is found to be still quite clear at a time when the euglobulins of the globulin fraction are to a large extent precipitated. Prolonged shaking however produces coagulation of some of the proteins in the albumin fraction, also the

smaller the quantity of salts present, the more rapidly does this occur. In any way, the amount of protein in the shaken albumin fraction found to be coagulated is very small compared with the big precipitate in the globulin fraction.

In order to decide, whether this coagulated protein of the albumin fraction is pseudoglobulin or albumin, these serum proteins were prepared and tested separately. I obtained these preparations by third, half and total saturation with the sulphate of ammonium, and the euglobulin as well by dilution of a serum and acidification with acetic acid up to its isoelectric point. The proteins thus obtained and freed from the ammonium sulphate by dialysis, were then shaken under varied conditions as to protein—or salt concentration. Summarising my results I can say :

The production of froth was well marked in the albumin solution, to a slightly lesser extent in the pseudoglobulin solution, but the euglobulin solution gave a froth only as long as shaking was going on and this froth being unstable quickly disappeared. This is interesting in regard to the different influence which these proteins exert upon the surface tension of water. Cloudiness followed by precipitation was first observed to occur in the euglobulin solution, then in the pseudoglobulin solution, and finally in the albumin solution, the latter requiring eight hours' shaking. Higher salt concentration tended to inhibit the precipitation, but this inhibition was less marked in the case of euglobulins.

In view of the great difficulty of getting absolute separation of the different proteins, I am far from considering the proteins, prepared as described, as being pure. I therefore repeated the experiments with preparations of albumin and globulins of horse serum, very pure preparations, which Dr Hartley kindly put at my disposal. I shall refer to these preparations later on in regard to the surface tension. The shaking effect of these proteins was similar to those described above, the albumins requiring the longest time to be precipitated. P. Schmidt (1914) did not evidently shake the albumin long enough, for he did not succeed in getting cloudiness and precipitation.

In spite of the certain restrictions which are to be observed in applying these observations to those observed when shaking the whole serum, I am inclined to consider it highly probable that the euglobulins in the serum are the first proteins which are denaturated and precipitated by shaking. Some alterations in the albumins must also have taken place, because their influence on the dispersity of the euglobulins must

have been lessened. If albumins either in the form of a CO_2 -albumin fraction, or in pure state, be added to the serum before shaking takes place, this excess of albumin prolongs the time necessary for the precipitation of the englobulin, and *vice versa* an excess of globulin is found to shorten this time. Only when a serum is shaken for a very great length of time can it be assumed that part of the pseudoglobulin as well as of the albumin is precipitated in a denaturated state.

In every case, where a precipitate was produced by shaking, the latter has been found to be insoluble except in alkalis, which shows that denaturation has taken place.

Now the question arises what is the real cause of this denaturation of the proteins, which occurs when they are shaken with a gas, and what other processes accompany this shaking-effect.

The effect of shaking explained as a consequence of change in surface energy.

The observation that shaking of a protein solution with a neutral gas produces the separation of a solid body, can be traced back to 1851, when Melsens and Harting first observed this phenomenon, which was later confirmed by Smee (1863) and more closely investigated by Plateau (1873), who considered the formation of solid surface membranes to be of a similar nature. The same observations were made by Naegeli (1880) and Kauder (1886). To Metcalf (1905) we owe a thorough investigation of the nature of the formation of surface membranes, especially those occurring in solutions of peptone. Peptone as well as other substances, the solutions of which tend to form surface membranes, lower the surface tension of water [Freundlich, 1909]. According to Gibbs' thermodynamic principle those substances will be positively adsorbed at the free surface of the liquid, thus producing a relatively higher concentration in the surface. This increase of concentration in the surface may become so great, that gel-formation takes place. The conception of H. Freundlich (1909, p. 79) is probably more correct, namely that the adsorbed substance in the surface is undergoing reversible or irreversible alterations in a less soluble body, especially if the substance is by itself not readily soluble in water as the dispersion medium. This change in solubility has also been observed by Deveaux (1904), who noticed the formation of white insoluble membranes, when egg albumin was dropped on to a clean surface of water. These phenomena are especially well marked in the case of egg albumin and

have been closely investigated by Ramsden (1894). It is evident, that a soluble substance tending to form surface membranes, will show this tendency more so, if by means of shaking the surface is considerably enlarged. Now Ramsden demonstrated this accelerating effect of shaking on the formation of surface membranes, and he succeeded in bringing about almost complete coagulation of the protein content of an egg white solution by means of shaking.

What happens now in the case of a shaken serum? *In vivo* the serum has no free surface against air, but is everywhere surrounded by a wall covered with endothelium. It is probable that the adhesion on this wall is in equilibrium with the cohesion of the serum, thus giving rise to no surface tension of the serum and therefore thus insuring the stability of the serum proteins. If the serum has however a free surface against air, the cohesion will be larger than the adhesion to the air, and a certain positive value of surface tension of the serum against air will result. Now the serum is an electrolyte containing protein hydrosol, and its surface tension against air is less than that of pure water, as J. Traube (1908) first showed. This lower value of surface tension is chiefly due to the presence of proteins. If according to Maraghini (1912) the protein substances are removed by filtration through a collodium membrane, the filtrate will show a surface tension similar to water and even a little higher, this fact being due to the presence of neutral salts, which increase the surface tension. The proteins show therefore a tendency to be positively adsorbed on the free surface against air and the more they lower the surface tension of their dispersion medium, the greater is the tendency for this to occur. Comparative experiments have shown that the loss of surface tension produced by the serum albumins is found to be somewhat higher than that caused by the globulins.

I give below in a table some data, with regard to the surface tension of the different proteins. The figures represent the number of drops given by a stalagmometer of Traube and calculated as mentioned in my former paper (this *Journal*, 1913, 316).

From these data it follows that a serum albumin tends to lower the surface tension of water against air more than the globulins do. This is in accordance with experiments made by F. Bottazzi (1913), but is contrary to the findings of Iscovesco (1910), who found a pure egg albumin to increase the surface tension of water, whereas only the globulins cause a loss of surface tension. Either egg or serum albumin behaves differently as regards its influence upon surface tension, which

						Heated $\frac{1}{2}$ h. at 56°	Boiled
I.	Water	100.00	—	—
	Serum undiluted	111.65	—	—
	Serum diluted 1:8 with aqu. dist.	102.51	—	—
	Albumin fraction not rendered isotonic ($\frac{1}{10}$)	102.77	107.31	111.02
	„ „ rendered isotonic ($\frac{1}{10}$)	104.95	105.05	112.56
	Globulin fraction in 0.85 % NaCl ($\frac{1}{10}$)	102.16	104.95	106.70
	Serum dil. $\frac{1}{20}$ with 0.85 % NaCl	102.67	104.49	109.83
II.	Serum diluted 1:4 with aqu. dist.	102.50
	Albumin fraction not isotonic ($\frac{1}{10}$)	104.30
	„ „ „ heated $\frac{1}{2}$ hour at 56°	105.81
	„ „ „ 2 hours shaken at 37° and filtered	103.39
	„ „ „ Control 2 hours at 37°	105.06
III.	Serum diluted 1:10 with NaCl solution	103.67
	„ „ „ „ heated $\frac{1}{2}$ hour at 56°	106.87
	„ „ „ „ 2 hours shaken at 37°	103.67
	„ „ „ „ 2 hours control at 37°	104.19
	Globulin fraction, well washed with aqu. dist. then diluted 1:5 with 0.85 % NaCl	101.4
	Globulin fraction, heated $\frac{1}{2}$ hour at 56°	101.75
	„ „ 1 hour shaken	101.55
	Albumin fraction dil. 1:8, not isotonic	103.97
	„ „ heated $\frac{1}{2}$ hour at 56°	106.06
	„ „ 2 hours shaken at 37° and filtered	101.22
	„ „ Control at 37°	103.95

Surface tension of horse serum preparations.

						Aqu. dist.—100
1.	50 c.c. aqu. dist.	+0.5 gr. air dried whole horse serum	103.15
2.	50 c.c. „	+0.5 gr. „ albumin	113.74
3.	50 c.c. „	+0.5 gr. „ globulins (eu.+ pseudoglob.)	107.08

would be very interesting, or the albumin is not free from the accidental presence of other substances, which lower the surface tension. These may be the serum pigments, which are naturally present in the CO₂-albumin fraction, and also in the albumin preparations obtained by salting out, for a total saturation with the sulphate of ammonium involves the pigments, these latter coming down with the precipitate. This question, viz. how far the observed loss in surface tension in albumin solutions is due to the actual accidental presence of such substances, I must leave open for the present, but for the sake of argument will assume it to be the case, namely that serum albumin produces a considerable loss of surface tension of the water against air.

Now a serum exposing a free surface to air is by that reason alone

rendered unstable, involving as it does the adsorption of albumins. This adsorption decreases the protecting influence which the albumins exert on the dispersity of the euglobulins in virtue of their greater influence on surface tension. This process is favoured by any increase of temperature which produces an increase in the intensity of the Brownian movement. The euglobulin thus becoming more labile tends to aggregation and precipitation, undergoing a slowly but steadily progressing denaturation. This process, which takes place when serum is exposed with a free surface to air, and which is accelerated by an increase of temperature, is accelerated to a considerable extent when, by means of shaking with air, not only is its free surface enlarged but also continuously changed and renewed. This continuous alteration of the surface causes the already aggregated protein particles to disperse again, which process, when continuously repeated, favours the condition of irreversible insolubility of the proteins, as has been already pointed out by Ramsden. The dispersion of already aggregated proteins is the more marked, the higher the protein concentration in the serum is, *i.e.* the more the mutual protecting influence of the different proteins can be exerted. Dilution of the serum favours therefore the occurrence of the condition of coagulation and irreversible insolubility. This irreversible change in solubility affects naturally first the euglobulins, which by themselves show the least tendency to solution. If the serum has been previously heated, which causes a considerable loss of surface tension, as observed by Traube (1908) and confirmed by many other authors, it may be possible to assume that the formation of capillary-active substances by heat, according to L. Berczeller (1913), albumoses and pepton, prevents the proteins from getting adsorbed on the surface, thus allowing them a greater stability against the influence of storage and shaking. This may also be taken into account when considering the influence of the electric charge of the denaturated protein particles upon their state of dispersion.

In a former paper of mine (1913) I was able to show that the surface tension of a serum is increased by shaking. The collected froth shows however lower surface tension than the shaken liquid, which is in accordance with Gibbs' principle.

How far the shaking effect upon a serum is due to alterations of the surface energy is best demonstrated by the fact that the addition to a serum of traces of such a capillary-active substance, as saponin is, completely prevents the proteins from being affected by the shaking, whatever the length of time during which it is shaken.

The possibility that, apart from alterations of the surface energy, other factors may play a more or less important part in the production of the shaking effect, cannot be excluded. It may be assumed, for instance, that the rapid movement of the air bubbles through the liquid gives rise to an electric charge on their surface, the density of which will increase with the curvature of the bubbles, *i.e.* with their smallness, and it may be possible that these electrically charged surfaces play a part in favouring the aggregation and coagulation of the proteins in solution. No definite evidence however is forthcoming to support this view.

Finally I mention the probability that a change in the H⁺-concentration in the shaken serum has taken place. Using neutral red and rosolic acid as indicators, I could not find however any difference in reaction of a shaken as compared with an unshaken serum. The addition of either traces of alkali or of acid to a serum proved to be without any inhibiting effect upon the effect of shaking, but I found that alkali produced a slight acceleration of the coagulation and precipitation in the shaken serum. In view of the importance of the H⁺-concentration of a serum as regards its serological properties, the question of any alteration of H⁺-concentration as a result of shaking deserves a special study.

GENERAL CONCLUSIONS.

Shaking a serum with air produces coagulation and denaturation of the euglobulin, due to alterations in the surface energy of the serum, leading to adsorption of albumin, thus rendering the euglobulins labile, which then coagulate and become insoluble.

This process is not due to oxidation, and it has been shown that conditions which render the euglobulin more stable, such as previous heating and the presence of a hypertonic medium, tend to inhibit the effect of shaking, whereas dilution with water or storage, rendering the euglobulins labile, favours also the effect of shaking.

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STUDIES ON THE INACTIVATION OF COMPLEMENT
BY SHAKING.

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INTRODUCTION.

IN a former paper on the inactivation of the haemolytic complement by means of shaking (1913), I considered it possible that the process of shaking-inactivation might be explained by assuming that an adsorption of the complement occurred on the surfaces of the precipitate, which is always found when a serum is shaken. I was unable however either to fix the complement of a fresh serum by adding the centrifuged coagula of a shaken serum or, on the other hand, to set free a complement presumably adsorbed on the surface of precipitated proteins, which are found to be soluble only in alkali. I therefore had to leave undecided the question as regards the nature of the process of shaking-inactivation.

About the same time and independently P. Schmidt and M. Liebers (1913), working on the shaking-inactivation of the haemolytic complement, came to the following conclusion: shaking of guinea-pig serum produces precipitation of the euglobulins followed by adsorption of the complement on these newly-formed surfaces. The visibly coagulated globulin is only a minimal part of the total globulin, and the shaken and centrifuged serum contains therefore not only globulins but also complement not yet adsorbed. They succeeded in bringing the globulin, precipitated by shaking, back into solution by a normal E-piece, thus setting free the complement adsorbed on these surfaces, enabling it to act again.

L. Hirschfeld and R. Klinger (1914), in their researches on the shaking-inactivation, worked with human serum and came to the conclusion that shaking produces alteration in the stability of the globulins, thus causing a part of them to coagulate. This alteration interferes










with complement action, and sera so treated become anti-complementary, giving for instance a positive Wassermann reaction. Recent work of A. Bessemans (1913), however, shows that human and guinea-pig sera are different as regards the stability of their globulins, and results, obtained with experiments on human serum, are therefore not to be compared with those obtained with guinea-pig serum except to a restricted degree.

I therefore resumed my investigations on the nature of the inactivation process by shaking chiefly with a view to ascertain in how far the conception of P. Schmidt could be adapted to the experimental facts.

I give below a record of experiments made from a serological point of view as regards the influence of shaking upon the haemolytic complement of guinea-pig serum, and consider them to be a continuation of my former work on the same subject.

Technique of experiments.

The technique employed in shaking the sera is principally the same as that indicated in my former paper (1913). Haemolysis was due to the complementing action of the serum in combination with sensitized sheep red corpuseles, of which a 2.5 % emulsion in 0.85 % saline was used. Sensitization was effected with inactivated rabbit immune-serum, the dose of which will be given in the protocols. The following scheme has been adopted to illustrate different degrees of haemolysis :

-  No haemolysis.
-  Faint trace of haemolysis.
-  Very slight haemolysis.
-  Well-marked haemolysis.
-  Half haemolysed.
-  Strong haemolysis.
-  Very strong haemolysis.
-  Nearly complete haemolysis [a slight trace of unlaked cells at the bottom].
-  Complete haemolysis.

Experimental part.

In another communication (1914), considering the effect of shaking upon a serum from a more general standpoint, I have shown that the effect of shaking upon a serum is due to alterations in the surface energy

involving changes of the serum albumins and denaturation of the serum proteins, which first of all affects the euglobulin. The process is to be considered as progressing with the time, till irreversible denaturation of the proteins is effected. The various stages of this process are serologically characterised by the steadily progressing loss of complement activity as indicated by the different procedures necessary to restore the complementing power. The different factors necessary to the restitution of complement activity have been already mentioned in my former paper (1913), but before I can attempt to explain these very complicated relations, I must first of all complete the description of my experiments.

Reactivation of a shaken serum by a fresh serum.

A serum inactivated by shaking can be reactivated by the addition of fresh serum. This reactivation can be effected under any conditions, and it is the better marked the more the shaken inactivated serum can be reactivated by other factors, as will later be shown. The question as to the restitution of a shaken serum by a normal serum is, in reality, how far the former interferes with the action of normal complement. Jakoby and Schuetze (1910) found no inhibition to take place, a fact which I can confirm. It proved to be the same, whether the precipitated globulins were removed by the centrifuge and filtering, or not removed at all. I found also that the precipitate itself showed no inhibiting effect on normal complement action. Only when present in large excess did slight inhibition sometimes occur. This is confirmed by the work of Landsteiner and Stankovic (1906), who found that heat-coagulated serum proteins produced a slight inhibiting effect. Hirschfeld and Klinger (1914), however, found that sometimes the centrifuged precipitate of a shaken serum exerted an anti-complementary effect, but this was not constant. On the other hand the sera after shaking were sometimes so anti-complementary as to give a positive Wa. reaction, even if the serum had been well centrifuged. But it must be remembered that these authors worked exclusively with human serum, which may behave very differently from guinea-pig serum when shaken, the latter showing no inhibiting effect after shaking, as also Courmont and Dufourt found (1912).

Whether inhibition of normal complement action takes place or not would according to P. Schmidt's conception be explained by assuming a partial or complete saturation of the adsorbing surface of the

precipitated globulins, but there seems to be some difficulty in understanding why the albumins of the fresh serum do not effect a solution of the globulins, thus setting the adsorbed complement free, such as occurs on the addition of a normal E-piece. It may be possible also to assume, that addition to a fresh serum of such a dispersed phase as an opalescent shaken serum represents gives rise in the fresh serum to adsorptions by increase of surface analogous to those which effected the inactivation by shaking.

The reactivation of a shaken serum by means of the complement fractions.

As regards the technique it must be mentioned, that in order to obtain the complement fractions, the CO₂-method of Liefmann has been employed, it giving more uniform results than the HCl-method of Altmann, which I had previously employed. After diluting the serum 1 : 8 with aqu. dist., CO₂ was passed through for half an hour, the temperature being kept low by means of ice. The serum was then well centrifuged, the supernatant fluid filtered through hardened paper and then rendered isotonic. The precipitate was washed once with distilled water and diluted with 0.85 % NaCl solution immediately before use.

For the sake of brevity I shall employ the following signs :

C_n = normal complement containing serum.

C_{sh} = serum inactivated by shaking at 37° C.

C_{th} = serum inactivated by heating at 56° C. for half an hour.

C₅, C₁₀, etc. = sera inactivated by heating at 56° C. for 5, 10, etc. minutes.

E_n and M_n = CO₂ fractions obtained from a normal serum (C_n).

E_{sh} and M_{sh} = CO₂ fractions obtained from a shaken inactivated serum (C_{sh}).

E_{th} and M_{th} = CO₂ fractions obtained from a thermo-inactivated serum (C_{th}).

E₅, E₁₀, etc. and M₅, M₁₀, etc. = CO₂ fractions from sera heated at 56° C. for 5, 10, etc. minutes.

Ritz (1912) showed that the process of inactivation by shaking consists of two stages. In the first stage it is possible to reactivate C_{sh} by M_n or E_n, in the second stage this has become impossible. I found previously the reactivation by M_n to be impossible, and if we are to understand by reactivation a complete restitution of an

otherwise completely inactivated shaken serum, I must still adhere to that opinion; but that a partial reactivation can be effected by M_n is to be seen from the experiments I and II.

Exp. I. Guinea-pig serum, diluted 1 : 10 with 0.85 % saline, was shaken for different times, then tested as regards its haemolytic power and its reactivation by M-piece and E-piece.

10 c.c. of this serum was shaken in tube of 60 c.c. volume.

Haemolysis of 0.5 c.c. sensit. red cells (AB dose = three times the single lysing dose) after 1 hour at 37° and 15 hours at room temperature.

				0.5 c.c. E_n 0.5 c.c.	0.5 c.c. M_n 0.5 c.c.	0.5 c.c. saline 0.5 c.c.
+ C_{sh}						
after being shaken at 37° for $\frac{1}{2}$ hr.				■	■	■
1 hr.				■	■	□
$1\frac{1}{2}$ hrs.				■	■	□
2 hrs.				□	□	□
+ M_n		■		□
+ E_n			■	□
+ C_n control serum						■
+ 0.85 % saline						□

Exp. II. C_n diluted $\frac{1}{10}$ with saline was shaken; 13 c.c. liquid in 67 c.c. containing tube.

Hours shaking time	$\frac{1}{10} C_{sh}$			0.5 c.c. of							
	1.0	0.5	0.25	M_n	E_n	C_5	C_{10}	C_{15}	C_{20}	C_{25}	C_{30}
C_{sh} $\frac{1}{2}$	■	■	■	■	■	■	■	■	■	■	■
1	■	■	□	■	■	■	■	■	■	■	■
$1\frac{1}{2}$	■	□	□	■	■	■	■	■	■	■	■
2	□	□	□	■	■	■	■	■	■	■	■
$2\frac{1}{2}$	□	□	□	■	■	■	■	■	■	■	■
$3\frac{1}{2}$	□	□	□	■	■	■	■	■	■	■	■
C_n ...	■	■	■	■	■	■	■	■	■	■	■
C_n control $3\frac{1}{2}$ h. at 37°	■	■	■	■	■	■	■	■	■	■	■
+ 0.5 c.c. E_n				■		■	■	□	□	□	□
+ 0.5 c.c. M_n					■	■	■	□	□	□	□

Every half an hour 1.5 c.c. was taken out, centrifuged and tested.

C_n was undiluted, exposed to 56° C. for between 5 and 30 minutes, then diluted 1 : 10 with saline.

(AB dose and amount of red cell emulsion equal to Exp. I.)

Haemolysis after 1 hour at 37° and 15 hours at room temperature.

To 0.5 c.c. of C_{sh} was added 0.5 c.c. of M_n , E_n or C_n , the total volume being 1.5 c.c.

These experiments I and II, as well as many others giving similar results, demonstrate that a serum, the complementing power of which is considerably diminished by the shaking, but which is yet not completely inactivated, can be fully restored by M_n . If however the inactivation process is further advanced and finally completed, the haemolytic action of the serum can no longer be restored by M_n , except to a very small degree, while E_n has still its full reactivating effect. Further shaking of the serum will diminish and finally abolish the possibility of reactivation by E_n , and, as Exp. II shows, also that of C_{th} . Therefore the inactivation process by shaking is to be considered as progressing in such a manner that the possibility of reactivation by M_n is first lost, later that of E_n , and finally the reactivation by C_{th} disappears. Such a serum is then irreversibly inactivated.

The reactivation of a serum inactivated by shaking, by means of a thermo-inactivated serum and the relation of this process to the reactivation by the normal complement fractions.

The reactivation of C_{sh} by C_{th} is in some way connected with the reactivation by means of M_n and E_n , but before attempting to explain this, I will give the results of a series of experiments demonstrating these facts.

The above-mentioned Exp. II shows that the possibility of reactivating C_{sh} by C_{th} decreases progressively, either if the C_{th} is heated for a longer and longer time, or if the C_{sh} is submitted to a more prolonged shaking.

In a former paper (1913) I pointed out that, generally speaking, C_{sh} can be reactivated by C_{th} as long as the latter can be reactivated by E_n . I shall refer to this later. In Exp. II however this is only approximately the case, for it is shown that C_{15} is able to reactivate a C_{sh} shaken 2 hours, to a large extent, in spite of the fact that neither E_n nor M_n has any effect on C_{th} . This is exactly what Ritz explains by assuming a third component.




































Exp. III. A guinea-pig serum of 24 hours standing was partly shaken at 37° undiluted, partly heated at 56° for half an hour and partly not treated.




















All sera were then submitted to the effect of CO_2 and thus the fractions were obtained, the signs for which are employed as above stated.

Haemolysis of 1 c.c. sensit. red cell emulsion after 1 hour at 37° and 15 hours at room temperature.

(AB dose = four times the single lysing dose.)

Exp. III shows that C_{sh} can be reactivated by M_n , the more so the greater the dilution of M_n , and that M_{30} , i.e. a M-piece derived

	c.c.	1.0	0.5	0.25	0.15	0.1	0.0
$\frac{1}{10} C_n$							
„ C_{sh}							
„ C_{30}							
1 c.c. $\frac{1}{10}$ diluted E_n + 1 c.c. 0.85 % saline							
„ $\frac{1}{20}$ „ M_n + „ „ „							
„ $\frac{1}{10}$ „ E_n + „ $\frac{1}{20}$ diluted M_n							
„ „ „ E_{30} + „ 0.85 % saline							
„ „ „ M_{30} + „ „ „							
„ „ „ E_{30} + „ $\frac{1}{10}$ diluted M_{30}							
„ „ „ E_{sh} + „ 0.85 % saline							
„ „ „ M_{sh} + „ „ „							
„ „ „ E_{sh} + „ $\frac{1}{10}$ diluted M_{sh}							
1 c.c. $\frac{1}{10}$ diluted E_n							
„ „ C_{30} + „ „ E_n							
„ „ C_{30} + „ „ C_n							
„ „ C_{30} + „ „ C_{sh}							
„ „ C_{30} + „ „ E_{sh}							
„ „ C_{30} + „ „ M_{sh}							
„ „ M_n + „ „ E_{sh}							
„ „ E_n (heated) + „ „ M_{sh}							

1 e.c. γ_0^1 diluted	$C_{sh} + 1$ e.c. $\frac{1}{2}$ diluted	M_n	...			
"	"	" + "	$\frac{1}{4}$ " "	...		
"	"	" + "	$\frac{1}{20}$ " "	...		
"	"	" + "	$\frac{1}{2}$ " "	M_{sh}	...	
"	"	" + "	$\frac{1}{4}$ " "	" "	...	
"	"	" + "	$\frac{1}{10}$ " "	" "	...	
"	"	" + "	$\frac{1}{2}$ " "	M_{30}	...	
"	"	" + "	$\frac{1}{4}$ " "	" "	...	
"	"	" + "	$\frac{1}{10}$ " "	" "	...	
"	"	" + "	$\frac{1}{10}$ " "	E_n	...	
"	"	" + "	$\frac{1}{20}$ " "	" "	...	
"	"	" + "	$\frac{1}{40}$ " "	" "	...	
"	"	" + "	$\frac{1}{10}$ " "	E_{sh}	...	
"	"	" + "	$\frac{1}{20}$ " "	" "	...	
"	"	" + "	$\frac{1}{40}$ " "	" "	...	
"	"	" + "	$\frac{1}{10}$ " "	E_{30}	...	
"	"	" + "	$\frac{1}{20}$ " "	" "	...	
"	"	" + "	$\frac{1}{40}$ " "	" "	...	
"	"	" + "	$\frac{1}{10}$ " "	E_n (heated)	...	

from a thermo-inactivated serum, gives a more complete reaction than M_n , M_{sh} having no effect.









































In the case of the reactivation by E-piece it is irrelevant whether the E-piece is derived from a normal or from a thermo-inactivated serum. Even the addition of E_{sh} has a marked effect.

The thermo-inactivated serum, C_{30} , was not anti-complementary nor could it be reactivated by E_n or by E_{sh} , except to a very slight extent. On the other hand its reactivating power on C_{sh} was well marked, the latter being however not completely inactive.

Exp. IV. Fresh guinea-pig serum was treated with CO_2 , and the M-piece thus obtained shaken for 1 hour at 37° ; other parts of the M-piece were diluted in different concentrations and then either heated or not treated otherwise.

Each tube contains 1 c.c. $\frac{1}{10}$ diluted C_n. M-piece in decreasing amounts, saline to fill up to 2 c.c. and 1 c.c. sensit. red cell emulsion. (AB dose = three times the single lysing dose.)

Haemolysis after 1 hour at 37° and 24 hours standing at room temp. The control reactions were satisfactory.

				c.c.	1.0	0.5	0.25	0.15
M _n 1:2 diluted					
„ 1:5 „					
„ 1:10 „					
„ 1:20 „					
$\frac{1}{10}$ diluted M _n 1 hr. shaken at 37°	...							
M _n 1:5 diluted, heated 30 mins. at 56°								
„ 1:10 „ „ 10 „ „								
„ „ „ „ 20 „ „								
„ „ „ „ 30 „ „								
„ 1:20 „ „ 30 „ „								

Exp. IV shows the anti-complementary effect of a normal M-piece on a normal serum, decreasing with dilution and with heating, the shaken M-piece having no longer any anti-complementary effect.

Exp. V. Haemolysis after 1 hour at 37° and 15 hours standing at room temp.

1 c.c. sensit. red cell emulsion, AB dose being three times the single lysing dose.




























Total volume in each tube = 3 c.c., of each factor 1 c.c. being in reaction.

From Exp. V it follows that a shaken serum which is completely inactivated and unable to be reactivated by a normal M-piece is restored by the addition of a M-piece obtained from a thermo-inactivated serum.

The collected froth of a shaken serum retains its haemolytic power longer than the shaken liquid.

No difference is to be found in the reactivation of a shaken serum by an E-piece, whether obtained from a normal serum or from a thermo-inactivated serum.

In the course of thermo-inactivation the property of being reactivated

1 : 10 diluted	E _n	+	saline	...	
..	M _n	+	
..	E _n	+	M _n $\frac{1}{10}$ diluted		
..	E ₅	+	saline	...	
..	M ₅	+	
..	E ₅	+	M ₅ $\frac{1}{10}$ diluted		
..	E ₃₀	+	saline	...	
..	M ₃₀	+	
..	E ₃₀	+	M ₃₀ $\frac{1}{10}$ diluted		
..	M _n	+	E ₅		
..	..	+	E ₃₀		
..	E _n	+	M ₅		
..	..	+	M ₃₀		
..	C _n	+	M _n $\frac{1}{2}$..		
..	..	+	M ₅		
..	..	+	M ₃₀		
..	C _{sh}	+	M _n		
..	..	+	.. $\frac{1}{10}$..		
..	..	+	M ₅ $\frac{1}{2}$..		
..	..	+	.. $\frac{1}{10}$..		
..	..	+	M ₃₀ $\frac{1}{2}$..		
..	..	+	.. $\frac{1}{10}$..		
..	..	+	saline	...	
..	collected froth of C _{sh}	+	
..	C _{sh}	+	E _n $\frac{1}{10}$ diluted		
..	..	+	E ₅		
..	..	+	E ₃₀		

by the normal complement fractions is lost quicker in the case of the M-piece than in the case of the E-piece.



































The anti-complementary effect produced on normal complement by

a M-piece obtained from a thermo-inactivated serum is very small when compared with the inhibiting effect of a normal M-piece.

Exp. VI. Haemolysis after 1 hour at 37° and 15 hours at room temperature.

1 c.c. sensit. red cell emulsion. AB dose = three times the single lysing dose.

Total volume in each tube = 3 c.c., of each factor 1 c.c. being present.

						C _{sh}	E _n	Saline
1 : 5	diluted	M _n			⋮
1 : 10	"	"			
1 : 20	"	"			
1 : 5	"	M _n heated 10 mins. at 56° C.						⋮
1 : 10	"	"	"	"	"			
1 : 20	"	"	"	"	"			
1 : 5	"	M ₁₀			⋮
1 : 10	"	"			
1 : 20	"	"			
1 : 5	"	M ₁₀ heated 10 mins. at 56° C.						⋮
1 : 10	"	"	"	"	"			
1 : 20	"	"	"	"	"			
1 : 10	"	E _n			⋮
"	"	E _n heated 10 mins. at 56° C.						
"	"	E ₁₀			
"	"	E ₁₀ heated 10 mins. at 56° C.						
"	"	E _n			
"	"	E ₁₀			
"	"	C _n			
"	"	C ₁₀			
"	"	C _{sh} at 37°			
"	"	C control at 37°			
















This experiment shows first of all the great difference in the action of a M-piece upon an E-piece or on a shaken inactivated serum. Exp. VI shows also what we have already seen in Exp. V, that a shaken inactivated serum, not being reactivated by a normal M-piece, will become so, if the M-piece is derived from a thermo-inactivated serum, exposed to 56° C. for 10 minutes. Such a M-piece has a slight effect, when combined with a normal E-piece, but while heating such a M-piece will completely destroy this effect, the action of it on a shaken serum is but slightly diminished.

The same experiment shows further, that the effect of heating an isolated E-piece will not altogether destroy its power of reactivating a shaken serum, but it will do so, if the E-piece has been obtained from a serum already heated for 10 minutes at 56° C.

It is of interest to note in this connection, that Exp. III shows that a M-piece obtained from a shaken but not completely inactive serum acted quite well with a normal E-piece, but did no longer do so if the latter had been heated.

Exp. VII. Haemolysis of 1 c.c. sensit. red cell emulsion (AB dose = three times the single lysing dose) after 1½ hours at 37°.

			$\frac{1}{10}$ diluted E _n , c.c.	1.0	1.0	1.0	1.0	0.0
			0.85 % saline, c.c.		0.5	0.75	0.85	1.0
			+ c.c. ...	1.0	0.5	0.25	0.15	1.0
$\frac{1}{10}$ diluted M _n (of C _n)		■	■	■	◐	□
„ „ M _n shaken at 37°		□	□	□	□	□
„ „ M _n control at 37°		■	◐	◐	□	□
„ „ M _{sh} (of C _{sh})		■	■	◐	◐	□
„ „ M _n (of C control)		■	■	■	◐	□
			$\frac{1}{10}$ diluted M _n , c.c.	1.0	1.0	1.0	1.0	0.0
			0.85 % saline, c.c.		0.5	0.75	0.85	1.0
			+ c.c. ...	1.0	0.5	0.25	0.15	1.0
$\frac{1}{10}$ diluted E _n		■	■	◐	◐	□
„ „ E _n shaken (isotonic) 37°		■	■	◐	◐	□
„ „ E _n „ (not isotonic) 37°		■	■	◐	◐	□
„ „ E _n control (isotonic) 37°		■	■	◐	◐	□
„ „ E _n „ (not isotonic) 37°		■	■	◐	◐	□
„ „ E _{sh} (of C _{sh})		■	◐	◐	□	□
„ „ E _n (of C control)		■	■	■	◐	□

	0.85 % saline, c.c.		0.5	0.75	0.85	0.9	
	+ c.c.	1.0	0.5	0.25	0.15	0.1
$\frac{1}{10}$ diluted C_n					
„ „ C_{sh} at 37					
„ „ C control at 37					

From the observations made in Exp. VII the following conclusions can be drawn :

The normal action of a M-piece on an E-piece is abolished by shaking the isolated M-piece. If the M-piece however is derived from a shaken serum, the complementing power of which is considerably weakened by shaking, such a M-piece still has a well-marked effect with a normal E-piece.

On the other hand, if the action of a normal E-piece on a normal M-piece is considered, no loss of activity of the E-piece is observed when the latter has been shaken for an equal length of time as the M-piece.

[Only shaking the E-piece for a very long time will destroy its action.] The E-piece of a shaken serum is altered only to a very slight extent.

Exp. VIII. Fresh guinea-pig serum $\frac{1}{10}$ diluted was shaken 4 hours at 37°, then centrifuged and submitted to the following tests :

Haemolysis of 0.5 c.c. sensit. red cell emulsion (AB dose = twice the single lysing dose) after 1 hour at 37° and about 15 hours at room temperature.

$E_n B$ = E-piece, which after being rendered isotonic, was filtered through a Berkefeld filter (40 × 14 mm.).

$E_n A$ = E-piece, the reaction of which has been rendered equal to that of the C_n by means of addition of $\frac{n}{250}$ NaOH, neutral red and rosolic acid being used as indicators.

From Exp. VIII it follows that the reactivation of a shaken inactive serum by means of an E-piece is only slightly diminished if the latter has been filtered through a Berkefeld filter. [This observation is not constantly found, cf. Exp. IX.] But the action of the E-piece on a M-piece is totally abolished by the Berkefeld filtration.

A normal E-piece reactivates a shaken serum even after being heated 15 minutes, but a slight addition of alkali to the E-piece destroys its action, whether heated or unheated, on a shaken serum as well as on a normal M-piece [not constantly found].

1 c.c. $\frac{1}{10}$ diluted C _{sh}	+	2 c.c. $\frac{1}{10}$ diluted E _n	■
"	"	" + 1 "	"	E _n	■
"	"	" + 2 "	"	E _n B	■
"	"	" + 1 "	"	E _n B	■
"	"	" + 2 c.c. $\frac{1}{20}$	"	M _n	□
"	"	" + 1 "	"	M _n	□
"	"	" + 2 c.c. $\frac{1}{10}$	"	E _n heated for 15 mins. at 56° C.	■
"	"	" + 2 "	"	E _n A	"	"	□
"	"	" + 2 "	"	C _n	"	"	■
"	"	" + 2 "	"	E _n A	□
"	"	" + 1 "	"	E _n A	□
1 c.c. $\frac{1}{20}$ diluted M _n	+	1 c.c. $\frac{1}{10}$ diluted E _n	■
"	"	" + 1 "	"	E _n B	□
"	"	" + 1 "	"	E _n A	□
"	"	" + 1 "	"	E _n heated for 15 mins. at 56° C.	□
"	"	" + 1 "	"	E _n A	"	"	□
1 c.c. $\frac{1}{10}$ diluted C _{sh}	+	1 c.c. saline	□
"	"	C _n	+	"	■
"	"	C _{sh} (15 mins. at 56°) +	"	"	□

Haemolysis of 0.5 c.c. sensit. red cell emulsion after 1 hour at 37° and 15 hours at room temperature; sensitization effected with three times the single lysing dose of AB.

Exp. IX shows that a shaken serum can no longer be reactivated by an E-piece, if submitted to Berkefeld filtration. Contrarily to Exp. VIII this experiment shows that the E-piece after Berkefeld filtration was no longer capable of reactivating a shaken serum.

If an E-piece is heated for too long a time its action on a shaken serum will disappear.

Addition of alkali to an E-piece has a somewhat lesser inhibiting effect in this experiment than in Exp. VIII.









































































A thermo-inactivated serum, in spite of being reactivated by an E-piece, showed no action on the shaken inactive serum.

Experiment IX.

$E_nA = 0.5$ c.c. $E_n + 0.3$ c.c. $\frac{11}{250}$ NaOH in 0.85 % NaCl in each tube.

$E_nA_{th} = 0.5$ c.c. $E_n + 0.3$ c.c. $\frac{11}{250}$ NaOH in 0.85 % NaCl, the mixture heated for 30 mins. at 56° C.

NaCl = 0.5 c.c. NaCl solution + 0.3 c.c. $\frac{11}{250}$ NaOH in 0.85 % NaCl in each tube.

	E_n	E_nB	E_nA	E_{th}	E_nA_{th}	C_{th}	M_n	NaClA	NaCl
0.5 c.c. $C_{sh} \frac{1}{10}$ diluted, 1 hr. shaken, cloudy, being not filtered									
0.5 c.c. $C_{sh} \frac{1}{10}$ diluted, 2 hrs. shaken, not filtered (cloudy)									
0.5 c.c. $C_{sh} \frac{1}{10}$ diluted, 2 hrs. shaken, filt. through hard paper (opalesc.)									
0.5 c.c. $C_{sh} \frac{1}{10}$ diluted, 2 hrs. shaken, filt. through Berkef. filt. (clear)									
0.5 c.c. $C_{sh} \frac{1}{10}$ diluted, 3 hrs. shaken, not filtered									
0.5 c.c. $M_n \frac{1}{10}$ diluted									
0.5 c.c. C_{th} (30 mins. at 56°) $\frac{1}{10}$ diluted									
0.5 c.c. C control serum $\frac{1}{10}$ diluted, (3 hours at 37°)									

Summary of the experimental results.

In order to better survey the results obtained in the experiments described, I summarise them as follows :

A shaken serum can be reactivated by M_n , E_n , C_{th} or not at all, according to the degree of inactivation produced by the shaking [I, II].

The restitution effect of M_n is only found if the activity of the serum has not yet been completely destroyed [I, II]. This action of M_n disappears when the isolated M_n has been shaken or heated [VI], but a M-piece of a C_{th} may still exert full reactivation on a C_{sh} , when M_n no longer produces such an effect [V, VI]. Even heating a M_{th} will influence but little its restitution effect on a C_{sh} [VI]. Normally M_n has an anti-complementary effect upon C_n [IV]. This effect will be diminished by increasing dilution of the M_n and with the influence of heat upon it, but it will altogether disappear if M_n has been shaken [IV]. The normal action of M_n upon E_n will also be destroyed either by shaking or heating of the M_n [VII]. A M_{th} however still exerts some action upon E_n , which promptly disappears, if such an isolated M_{th} is exposed to heat. But even then it retains some of its reactivating power on a C_{sh} [VI].

A shaken serum completely inactivated as regards complement action can still be reactivated by E_n or C_{th} , when M_n will no longer have any effect [I, II]. It requires a much longer time to render an E_n inactive by shaking [VII] than it does for a M_n . An E_n works equally well as regards reactivation of C_{sh} , whether obtained from a C_n or C_{th} [V, VI]. If an isolated E_n be heated, its power of reactivating a shaken inactive serum is maintained for a longer time than its activity with a M_n [VIII]. The addition of alkali to an E_n , which obtained by CO_2 is acid in character, tends to inhibit its restitution effect on C_{sh} , but this result has so far not been obtained with regularity.

The reactivation of C_{sh} by means of C_{th} can take place, in spite of C_{th} not being able to be reactivated by M_n or E_n . The property of C_{th} to reactivate a C_{sh} decreases with a prolonged exposure to heat. *Vice versa*, however, a C_{th} may be reactivated by an E_n and yet be without effect on C_{sh} [IX]. In such a case no anti-complementary effect of C_{th} on a normal C_n could be observed [II].

By means of Berkefeld filtration it is possible to render a C_{sh} no longer able of being reactivated by E_n [IX], and on the other hand to allow an E_n still to retain its action on C_{sh} [VIII].

Theoretical part.

First of all I think it certain that shaking produces a progressing alteration of all the serum proteins, leading finally to their denaturation. This alteration affects principally and first of all the euglobulin, changes in the albumin and pseudoglobulin following later, but always being present before visible coagulation takes place. There is not yet enough evidence to identify the complementing property of a serum with a certain definite state of equilibrium in the physical conditions of the different proteins, as regards their quantity and their degree of dispersity, which latter are closely connected with the amount of salts present and the H⁺-concentration. But it can be safely assumed that complement action, whether due to an existing substrate or not, is dependent on certain physical conditions of the serum and will therefore become lost as a consequence of anything which alters these conditions. The reactivation of a serum is only a question of how far these alterations are reversible or not.

Now, the following explanation may cover at least the majority of observations, if not all.

Addition of a M-piece to a shaken serum can only produce reactivation if the alteration, which the albumins undergo by shaking, is not yet so far advanced as to prevent them from controlling the dispersity of euglobulin. It is evident that irreversible changes in the added M-piece brought about by shaking or heating destroy any effect which the addition of a M-piece may otherwise have. Alterations in the albumins in a serum produced by shaking take place more rapidly than the denaturation of the euglobulin, which latter process is only the consequence of the former, as I explained in greater detail in another communication [1914].

From this it follows that reactivation by M-piece very soon disappears, and that addition of an E-piece has an effect only so long as there are euglobulins still in a reversible condition of commencing denaturation. Their removal by a Berkefeld filtration abolishes any reactivation by an E-piece, but the Berkefeld filtration of an E-piece is not necessarily followed by a loss of its restitution power, for I found that Berkefeld filtration chiefly retains the pseudoglobulin of the E-piece (1914). Such a Berkefeld filtered E-piece shows no longer any activity with a normal M-piece, which is difficult to explain. This may be due to the absence of pseudoglobulins, which according to the recent work of Browning and Mackie (1914) represent the main factor in the haemolytic complementing action of a serum. I could however not obtain these results with well dialysed preparations of the different proteins of guinea-pig serum, so I must leave this question undecided. On the other hand, I found that addition of pure albumin obtained by fractionated salting out with the sulphate of ammonium has a similar but weaker effect on a shaken inactive serum than that of an E-piece [not constantly found]. To destroy this power of E-piece by shaking it requires a longer time, owing to its solubility in water being greater than that of the euglobulin.

If the shaking effect however is so advanced that no more euglobulin is left in a reversible state, the addition of both, E-piece as well as pure albumin, will have no more effect. If this be the case, sometimes a thermo-inactivated serum is found to have still a marked effect on the reactivation of a shaken serum, but before attempting to explain this fact the alterations of a fresh serum produced by heat must be considered.

As I described in greater detail in another communication, heating a serum to 56° C. for half an hour is followed by more or less marked denaturation of its proteins [H. Chick and C. J. Martin, 1913]. The H⁺-concentration is decreased and the heat denaturated particles are

kept in dispersion by their negative charge. If by acidification with CO_2 the particles become isoelectric with the medium, they aggregate and form a precipitate. The precipitate thus obtained (M_{th}) contains not only euglobulin with some pseudoglobulin, which form the precipitate by CO_2 in the case of a normal serum diluted with water, but it contains that portion of all proteins which has been affected by the heat. In redissolving the precipitate in saline, a part of it is often found to be insoluble, probably consisting chiefly of euglobulin. Such a M_{th} due to its albumin content can still reactivate a shaken serum upon which a normal M-piece will no longer have any effect. Perhaps it is the electrical charge of its particles and also its albumin content which renders such a M_{th} more resistant to heat than a normal M-piece.

If a serum is exposed to 56°C. , its complementing power rapidly decreases with the length of time, and this happens a little sooner in the case of an undiluted serum, due probably to the ratio of the protein content to the salt concentration being higher in the case of a diluted serum, and to the fact that the presence of salts lower the coagulation rate by heat [Chick and Martin].

If the time of exposure to 56°C. be varied, and the different samples of sera so obtained be treated by CO_2 in order to obtain the M-piece or E-piece, it is found by their combination with the corresponding fractions of a normal serum [cf. Exp. V] that first the E-piece, later the M-piece, is destroyed, *i.e.* that an action of a M_{th} in combination with an E_n can be obtained for a greater length of time than the action of an E_{th} with a M_n . This has been already found by many other authors [Sachs, 1913]. From this it follows that as long as a shaken inactivated serum can be reactivated by a normal E-piece, it can also be reactivated by a thermo-inactivated serum, if the latter is capable of being reactivated by an E-piece. In some cases however such a thermo-inactivated serum is found to have no influence on a shaken inactivated serum, in spite of both being reactivated by a normal E-piece. I think that alterations in the H^+ -concentration account for this phenomenon.

On the other hand, a thermo-inactive serum, incapable of being restored by an E-piece, may still reactivate a shaken serum. I assume that such a thermo-inactivated serum may have the faculty of bringing even the denaturated euglobulins back into solution, perhaps owing to its alkalinity. A similar faculty of lysing a precipitate is attributed to a heated serum by Welsh and Chapman (1909) in explaining their observation of the inhibition which a heated serum exerts on specific precipitation. If however the denaturation of the shaken proteins has

advanced too far, even this lysing effect of a heated serum can no longer be obtained.

There are other observations more difficult to explain, for instance the fact that a M-piece of a serum, heated for 10 minutes at 56°C ., gives a better reaction with a shaken serum than with a normal E-piece [Exp. VI], and further, that 10 minutes heating of such a M_{th} will abolish the effect on an E-piece but only slightly diminish the action with a shaken serum. I think it very probable that here again alterations in the H^+ -concentration play the most important part. Some preliminary investigations have shown that addition of traces of acid or alkali do not inhibit the effect of shaking, and on the other hand I found it impossible to reactivate a shaken serum by means of decreasing or increasing its H^+ -concentration. I intend however to continue my research on these lines, since I can confirm Noguchi's and Bronfenbrenner's observation, that it is possible only by means of adding some alkali to an E-piece, obtained by Liefmann's method, to restore full haemolytic action. But I shall deal with this in a later communication.

As already mentioned, P. Schmidt considers complement to be a ferment, an entity, which is adsorbed on the globulin surfaces in all processes, leading to their precipitation. I readily recognise how easily the main features of the processes involved in the shaking inactivation can be explained by this conception, but there are many points, the explanation of which by this hypothesis seems to be very difficult if not impossible. The following are some of these points :

P. Schmidt's conception does not explain the reason why the albumins in the shaken serum fail to bring the denaturated particles into solution, whereas fresh albumin will do so. Further the reactivation of C_{sh} by C_{th} , the fact that M_{th} has a reactivation effect on C_{sh} , whereas M_n is ineffective, and that such a M_{th} after being heated loses its activity with E_n but not with C_{sh} . The observation that an E_n being submitted to Berkefeld filtration remains active with a C_{sh} , but not longer with a M_n .

Last but not least the observation that a serum, submitted to the effect of dilution and standing, becomes inactive, if rendered isotonic, in spite of the euglobulins being in complete solution, etc.

I claim on the other hand to have shown, that at least as many of the observations can be equally well explained by assuming no substrate for a complement at all, but the necessity of certain physical conditions. I hope later to be able to bring forward more evidence in support of this conception.

GENERAL CONCLUSIONS.

It has been attempted to show that at least most of the experimental results, a summary of which is already given above, can be explained by assuming that shaking produces alterations of the physical conditions in the serum, which by themselves account for the loss of complementing power of the serum, and that the conception of a complement being a ferment, the action of which is due to the presence of a certain substrate, is not necessary. But there are some observations left unexplained by either conception, due to lack of further experimental evidence.

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STUDIES ON THE BERKEFELD-FILTRATION OF COMPLEMENT.

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INTRODUCTORY.

IN view of the divergent results obtained in recent work dealing with the effect of Berkefeld filtration on the haemolytic complement of serum, I think that the publication of the results which I have obtained during some investigations, which I have been carrying out on the inactivation of complement by shaking, may not be without value.

Muir and Browning (1909) found that by filtration through a Berkefeld filter the complement of a guinea-pig serum is retained by the filter to a large extent—sometimes indeed completely,—but if more serum is filtered it passes through. Neufeld and Andrejew (1909) and Andrejew (1910) found that out of all immune bodies in serum, complement is the most completely retained by filtration through Kieselguhr. Andrejew showed further that the relative loss of complement was considerably increased when the serum was previously diluted, no more complement passing through if the serum was diluted 1 : 10. A detailed investigation of the filtrability of complement was then undertaken by P. Schmidt (1911, 1912). He confirmed the observation of Andrejew in regard to the effect of dilution. His explanation was that the colloidal complement ferment, which he considers to be an entity, becomes less dispersed by dilution and therefore more easily retained by the filter. If undiluted, the serum proteins, especially the albumins, have a protecting influence upon the complement, which passes through the filter in the course of prolonged filtration, because the albumins cover the whole Kieselguhr

surface, thus shutting off the adsorbent filter surface from the complement. When he filtered the same serum repeatedly through the same filter, he found a decrease of proteins corresponding with a steadily increasing complementing power of the filtrate.










The results of my first experiments proved to be very contradictory, and not until I had made the technique as uniform as possible did I get uniform results, which could be reproduced under given conditions. There is no doubt that in the filtration of serum an adsorption on the filter material takes place, and it is therefore evident that according to the relations between filter surface and the filtrans different results can be obtained. I think it therefore necessary to have the size of the filter stated as well as the quantity of the filtrans. The fact that these data are wanting in most papers, renders a comparison of the experiments difficult, and on the other hand may explain the divergent results which the literature contains. That *ceteris paribus* also, the velocity of the filtration, dependent on the pressure, the H⁺-concentration, and the presence of neutral salts play an important part, will be shown at the end of this paper.

Technique of experiments.

The Berkefeld candles employed were cylinders of about 40 mm. height and 14 mm. diameter. The filtration was directed from outwards to inwards and was effected by a suction water pump having a pressure of about 680 mm. Hg.

During filtration the whole surface of the filter was kept covered by the liquid. On every occasion the filter was cleaned by filtering a hot diluted solution of NaOH followed by distilled water until the filtrate was neutral to phenolphthalein. In those cases where the filter was not completely dried before use (dist. water or 0.85 % saline remaining in the filter) the first portion of the filtrate was rejected. The complementary function of the serum was tested for its haemolytic power in combination with sensitized sheep red corpuscles, of which a 2.5 % emulsion in 0.85 % saline solution was used. The haemolytic immune-serum was inactivated rabbit serum, the single lytic dose of which was 0.00125 c.c. for 1 c.c. of the red cell emulsion and 0.1 c.c. complement containing serum.














































In the haemolytic tests the following schema has been adopted to illustrate different degrees of haemolysis.

-  No haemolysis.
-  Faint trace of haemolysis.
-  Very slight haemolysis.
-  Well-marked haemolysis.
-  Half haemolysed.
-  Strong haemolysis.
-  Very strong haemolysis.
-  Nearly complete haemolysis [a slight trace of unlysed cells at the bottom].
-  Complete haemolysis.

Experimental part.

Exp. 1. Filtration of fresh guinea-pig serum undiluted and 1:10 diluted with 0.85 % saline through a Berkefeld candle (50 × 14 mm.).

Haemolysis of 0.5 c.c. sensit. red cell emulsion, after 1 hour at 37° and 15 hours at room temperature. AB dose = 0.0025.

			C.c.	1.0	0.5	0.25	0.15	0.0
$\frac{1}{10}$ dil. compl. serum					
Undiluted through Berkefeld filtered, then 1:10 diluted with 0.85 % saline, one part of filtrate=3 c.c.	{	I						
		II						
		III						
		IV						
$\frac{1}{10}$ with saline diluted through Berkefeld filtered, each part of filtrate =10 c.c.	{	I						
		II						
		III						
		IV						

Exp. 1 shows that the loss of complementing power of undiluted serum seems to be relatively small compared with the nearly total loss in the case of the serum which has been diluted 1:10 with saline. It will be observed however that the absolute amount of native serum which passes the filter after filtration of 40 c.c. of the diluted serum is less than the amount of undiluted serum which has passed through the filter during the filtration of 6 c.c. undiluted serum. This experiment when repeated always gave similar results indicating that the relatively smaller loss of complement in an undiluted serum is only apparent.

If according to P. Schmidt the complement is supposed to be a ferment and an entity, it will be adsorbed on the Kieselguhr surface either

owing to its action on the surface tension or by electrical interaction [anomalous adsorption]. Adsorption is going on so long as the surface is not completely saturated, and saturation is more quickly reached in the case of undiluted serum. In the case of diluted serum much more liquid is required to saturate the adsorbing surfaces, so as to finally allow the complement to pass through. There is therefore no need to assume a lesser degree of dispersity of the complement ferment as an effect of dilution, for which there is also no other evidence. P. Schmidt supposes further that the complement already adsorbed on the filter surface will be freed in the course of the filtration by the protecting effect of the albumins, which accumulate at the filter surface. This seems to be difficult to understand, for there is no evidence that the albumins and the complement have a different action on the surface tension, and it is difficult to see why the albumins are not immediately adsorbed, thus preventing the complement from being adsorbed at all.





























































On the other hand it may be possible to assume that the complementing power of a serum is not bound on a certain substrate, but requires certain physical conditions of the serum, namely the relative state of the different serum proteins as regards their quantity and their degree of dispersity, which latter is closely connected with the amount of neutral salts present and the H⁺-concentration. If a serum which has complementing power is filtered through a Berkefeld filter, the whole filtering surface must first of all be changed by adsorption of proteins and its adsorptive power completely satisfied, before the serum can pass unchanged as regards its complementing power. This requires in a given filter a certain amount of proteins, and this condition of complete adsorption in the case of an undiluted serum is naturally more quickly reached. The first fraction of the filtrate is very poor in protein. With the increasing protein content the complementary function of the filtrate increases, finally reaching the original value of the serum before filtration. If such a serum is repeatedly filtered through the same filter, as P. Schmidt did 16 times, the protein content was found by him to steadily decrease, whereas the complement activity increased. The following Exp. 2 does not confirm this statement of P. Schmidt. But owing to the absence of detailed data in his paper the experiments can only be compared to a restricted degree.

Exp. 2. 30 c.c. of fresh undiluted guinea-pig serum was 17 times filtered through the same Berkefeld filter.

After each filtration 0.5 c.c. was taken and diluted 1 : 10 with 0.85 % NaCl.

Haemolysis after 1 hour at 37° and 1 hour at room temperature.

0.5 c.c. sensit. red cell emulsion. AB dose = 0.0025 c.c., the total volume being 1.5 c.c.

		1.0	0.5	0.25	0.15
Compl. serum (1%) normal					
Berkefeld filtrate	1				
	2				
	3				
	4				
	5				
	6				
	7				
	8				
	9				
	10				
	11				
	12				
	13				
	⋮				
	17				

Exp. 2 shows that the complementing power of the serum, which is still well marked after the first filtration, is slowly but steadily decreasing with the number of filtrations. After the 12th filtration had been effected, the complementing power was lost and did not reappear on further filtration. The first part of the first filtrate was almost free of protein as in Exp. 1. Thereafter the velocity of adsorption is asymptotically decreasing, *i.e.* so long as filtration is going on there will be adsorption but finally to an indefinitely small extent.

From Exp. 2 either a steadily progressing adsorption of complement ferment may be assumed or the relations of the proteins may be steadily changing till the alteration is such that complementing power is no longer possible.

The saturation of the filter surface can also be effected by the previous filtration of a thermo-inactivated serum as has been done by Muir and Browning (1909). A similar experiment was made by Holderer (1912), who effected the filtration of diastase through a Chamberland candle after the previous filtration of an albumin solution.





















In order to decide whether the different proteins in the serum undergo a different adsorption on the Kieselguhr surface of the filter, a series of experiments was made to show how far an inactive Berkefeld filtrate can be reactivated by the addition of the serum fractions which have been obtained by the CO_2 method of Liefmann. For the sake of brevity I call the fractions M- and E-piece respectively, the M-piece containing the euglobulin and a relatively small part of the pseudoglobulin, the E-piece containing all the albumin and the main bulk of the pseudoglobulin.

Browning and Mackie (1912) found that no reactivation either by M-piece or by E-piece takes place, but later (1913) they found a restitution possible but not with regularity. P. Schmidt showed that a filtrate which was ineffective by itself could be reactivated by a small amount of fresh serum which, though inactive by itself, produced reactivation by a summation of effects. In accordance with his own conception that the albumin fraction contains always traces of the complement ferment, the main bulk of which is adsorbed on the surfaces of the euglobulin precipitated by dilution and CO_2 , he concludes that a complete removal of the complement by means of adsorption on the filter is scarcely to be expected. The complement in the inactive filtrate is only in a state of subactivity. He found further that such a filtrate can replace an E-piece, *i.e.* that it can be reactivated by the M-piece.

Exp. 3. Reactivation of the filtrates of Exp. 1 by the fractions of the normal guinea-pig serum (CO_2 method).

Haemolysis after 1 hour at 37° and 18 hours at room temperature of 0.5 c.c. sensit. red cell emulsion.

AB dose = 0.0025 c.c. Total volume = 1.5 c.c.

		{ } filtrate				{ } ^{1/5} filtrate						0.85 %
		I	II	III	IV	I	II	III	IV	M-p.	E-p.	NaCl
(^{1/5})	0.5 c.c. E-piece											
(^{1/5})	0.5 c.c. M-piece											

Exp. 3 shows that in the case of the serum which has been filtered in the undiluted state no reactivation by any fraction can be obtained as long as the filtrate is inactive by itself. The filtrates of the diluted serum however could be reactivated by M-piece in an increasing degree, which means that with the progress of the filtration more albumin

passed through, which then increases the solubility of the M-piece. Such a Berkefeld filtrate acts therefore as an E-piece, just as P. Schmidt found. The addition of E-piece however to a Berkefeld filtrate of a diluted serum is not without effect. Either complement ferment passed through the filter in a state of subactivity, but becoming active by summation with the traces of complement ferment in the E-piece, or on the other hand the filtrate still contains globulins, which together with the globulins of the E-piece give a summation effect by the excess of albumin. In any way the restitution effect of the albumin fraction is very small.































In analogy with the result of Exp. 2, that in repeatedly filtering the same serum through the same filter a slow but steady decrease of complement occurs, the following Exp. 4 shows what happens if the same serum be filtered through a series of different filters.

Exp. 4. 20 c.c. of an undiluted fresh guinea-pig serum was filtered through a series of Berkefeld candles under similar conditions.

After each filtration 2 c.c. of the filtrate was removed, diluted 1 : 10 with 0.85 % saline and their haemolytic power as well as the effect of restitution by E-piece or M-piece investigated.

Control reactions with E-piece and M-piece worked satisfactorily.

Haemolysis of 0.5 c.c. sensit. red cells (AB dose = 0.0025 c.c.) after 1 hour at 37° and 15 hours at room temperature.

	1.0	0.5	0.25	0.0	1.0 c.c. E-piece + 1.0 c.c. filtrate	1.0 c.c. M-piece + 1.0 c.c. filtrate
$\frac{1}{10}$ normal compl. serum						
Filtrate N ₂ O						
I						
II						
III						
IV						

Exp. 4 shows that in this case the complementing power of the serum decreases rapidly with each filtration. The filtrate thus rendered inactive could not be reactivated by any means.

Filtration of the CO₂ fractions.

In connection with these experiments I undertook the isolated filtration of the CO₂ fractions and give in the following a record of some typical experiments.

*Filtration of Complement**Filtration of E-piece.*

Exp. 5. To decreasing amounts of M-piece diluted $\frac{1}{10}$ with 0.85 % saline 0.5 c.c. of filtered E-piece diluted $\frac{1}{10}$ is added.

Haemolysis after 1 hour at 37° and 1 hour at room temperature of 1 c.c. 2.5 % sensit. red cell emulsion. AB doses = 0.0025 c.c., the total volume in each tube being 2 c.c.

(1%) M-piece, c.c.		0.5	0.25	0.15	0.1	0.0
+ 0.5 (1%) E-piece		...	■	■	●	●
E-piece rendered iso- tonic after filtration	I	□				□
	II	□				
	III	□				
E-piece rendered iso- tonic before filtration	I	●	□	□	□	□
	II	■	■	■	■	□
	III	●	■	■	■	□





























Exp. 5 shows first of all the fundamental difference in the filtrability of an E-piece whether rendered isotonic or not. The passage of the E-piece was impossible before the addition of salt. The salted E-piece behaved similarly to a complement serum in so far as filtration is concerned, that is, the longer the filtration the more it comes through.

Muir and Browning have already found that the permeability of the Berkefeld filter is increased by the addition of salt to the serum, thus rendering the serum hypertonic. The serum is then brought back to the isotonic condition by the dilution with water. This has been later confirmed by Manol and Nowaczynski (1910). The last mentioned authors succeeded in regaining the complement retained in the filter by filtering immediately afterwards a hypertonic solution of NaCl. Muir and Browning did not succeed in this experiment. It must however be mentioned that Manol and Nowaczynski worked with the Chamberland filter.

Before attempting to give an explanation of the part played by the presence of salts, I will refer to another experiment, which shows this phenomenon.

Exp. 6. Effect of Berkefeld filtration upon complement serum diluted 1 : 10 with distilled water.

Haemolysis of 0.5 c.c. sensit. red cell emulsion (AB dose = 0.0025 c.c.) after 1 hour at 37° and 15 hours at room temperature.

	C.c.	1.0	0.5	0.25	0.15
Guinea-pig serum after standing 24 hours, diluted $\frac{1}{10}$ with 0.85 % NaCl					
The same serum but diluted $\frac{1}{10}$ with aqu. dist. After 4 hours standing at room temperature, there was very marked cloudiness, which completely disappeared by rendering the serum isotonic					
The serum thus treated and rendered isotonic + 1.0 c.c. ($\frac{1}{10}$) E-piece					
The serum thus treated and rendered isotonic + 1.0 c.c. $\frac{1}{10}$ M-piece					
After 4 hours standing at room temperature and before being rendered isotonic, the serum was filtered through a Berkefeld candle and the clear-looking filtrate rendered isotonic					
The isotonic Berkefeld filtrate + 1.0 c.c. ($\frac{1}{10}$) E-piece...	...				
The isotonic Berkefeld filtrate + 1.0 c.c. ($\frac{1}{10}$) M-piece...	...				

Exp. 6 shows first of all the influence of the dilution with aqu. dist. upon a complement containing serum. As I have pointed out in another paper (1914) the inactivation phenomenon of Sachs and Teruuchi (1907) by 1 : 10 dilution with aqu. dist., and standing a certain time, occurs only in absolutely fresh serum. The serum in Exp. 6 was 24 hours old and was not yet inactivated after 4 hours standing at room temperature when diluted 1 : 10 with aqu. dist. The complementing power however was lessened. The complete restitution by E-piece and the absence of such an effect by M-piece are in favour of some alteration of the albumins. If the diluted and cloudy serum after 4 hours standing, and before being rendered isotonic, was filtered through a Berkefeld filter, the serum became clear, just as if it had previously been salted, but if the filtrate was rendered isotonic it proved to be inactive and incapable of being reactivated by E-piece. The filtrate however still contained traces of euglobulin because the passing of CO_2 through it gave rise to a slight opalescence. Only if the serum stood for many days diluted 1 : 10 with aqu. dist. did I succeed in completely filtering off the euglobulin. As in Exp. 5 the absence of a sufficient amount of salts inhibited the passage of the albumins, for the restitution by the addition of fresh M-piece to the filtrate proved to be very small, but still better marked than the effect of E-piece.

A comparison of the different effects of filtration through paper or through a Berkefeld filter upon the action of E-piece is given in the following Exp. 7.

Effect of filtration upon E-piece.

Exp. 7. Guinea-pig serum after standing 24 hours in the cool room was diluted 1 : 8 with aqu. dist. and made acid by CO_2 .

The precipitate was once washed and immediately before use dissolved in 0.85 % NaCl in 1 : 20.

The supernatant fluid (E-piece) was treated in the following way :

























I. The supernatant fluid was rendered isotonic and remained otherwise untreated.

II. The supernatant fluid was filtered through soft filter paper and then rendered isotonic.

III. The supernatant fluid was filtered through hardened paper and then rendered isotonic.

IV. The supernatant fluid was rendered isotonic and then filtered through a Berkefeld candle.

Haemolysis of 0.5 c.c. sensit. red cell emulsion after 1 hour at 37° and 15 hours at room temperature. AB dose = 0.005 c.c., the total volume in each tube being 2.5 c.c.

$\frac{1}{20}$ M-piece	1.0	1.0	1.0	1.0	1.0	1.0	
NaCl sol.	0.0	0.5	0.75	0.85	0.9	1.0	Half saturation with
C.c.	1.0	0.5	0.25	0.15	0.1	0.0	ammonium sulphate
Of: I							Heavy precipitate.
II							" "
III							" "
IV							Only a slight dimness occurs, the liquid being almost clear.

Exp. 7 shows that a Berkefeld filtered E-piece is rendered incapable of giving an haemolytic effect with M-piece. The filtration removes about all the pseudoglobulins, which the albumin fraction contains, while the filtration through paper does not affect the pseudoglobulins. This is demonstrated by the effect of half saturation with sulphate of ammonium. In connection with this it is of interest to note that Browning and Mackie (1914) found recently that the pseudoglobulin represents the main factor as far as the complementing haemolytic power of a serum is concerned.








In regard to the filtration of the isolated M-piece, Exp. 6 shows that already without the presence of sufficient salts the euglobulins, being in suspension, are retained in the filter. Only a very minute portion passes with the diluted serum, probably owing to the small

amount of salts and to the protecting influence of the serum albumins. The filtration of the M-piece diluted 1 : 10 in 0.85 % saline has an effect similar to that of the filtration of the salted E-piece.

Exp. 8. M-piece obtained by the CO_2 method from a fresh guinea-pig serum was dissolved by 0.85 % NaCl solution in 1 : 10, and then filtered through a Berkefeld filter.

Each fraction of the filtration was of a volume of 10 c.c.

Haemolysis of 0.5 c.c. filtrate + 0.5 c.c. $\frac{1}{10}$ E-piece + 0.5 c.c. sensit. red cell emulsion (AB dose = 0.005 c.c.) after 1 hour at 37° and 15 hours at room temperature.

$\frac{1}{10}$ M-piece Berkefeld filtrate	I	II	III	IV	V	VI	VII	VIII
+ 0.5 c.c. E-piece $\frac{1}{10}$...							

Exp. 8 shows that the more prolonged the filtration the more M-piece passes through the filter. It behaves therefore just as the salted E-piece or the whole serum under similar conditions.

The effect of filtration upon the isolated fractions however must not be taken to represent what occurs if the two mixed together are filtered as in the case of a serum. Apart from other factors the different influence upon the surface tension of the medium by the different proteins would be already sufficient to vary the effect of filtration.

Theoretical part.

In attempting to give an explanation of all the phenomena observed in the Berkefeld filtration of serum it must be remembered that the filtration consists of several different processes, namely :

1. The mechanical retention of particles.
2. The adsorption on the filter material, in so far as surface tension is concerned.
3. The phenomena caused by the movement of the liquid in the capillaries of the filter causing either anomalous adsorption or coagulation.

The mechanical retention of particles occurred in the above described experiments only when the euglobulin was in suspension. It is however easy to demonstrate by means of an ultramicroscope the presence of many particles in the filtrate which escaped retention, but in view of the relatively large size of the Berkefeld filter pores (J. A. Craw, 1908) this is to be expected. It is also possible that in using a dry filter

the rushing of the liquid in the capillaries is so great that suspended particles may be carried through capillaries which otherwise would hold them back. This is shown by Grenet (1910) to occur in the case of filtration of bacteria. On the other hand, some of these small particles may consist of the filter material which appear especially when new filters are employed.

A more important effect upon filtration of serum is exerted by the adsorption of the Kieselguhr surface of the filter. According to Gibbs' thermodynamic principle those substances in the serum which lower the surface tension are adsorbed. These latter are principally the serum proteins, the albumins and the globulins. F. Bottazzi (1912), M. J. Gramenitzky (1913) and other authors have shown that the serum albumins as well as the serum globulins lower the surface tension of water, but the latter in a lesser degree. This may be the reason for the protective influence which the albumins have upon the stability of the dispersity of the globulins. I give below some data showing the influence of the Berkefeld filtration upon the surface tension of the filtered liquid. The figures represent the number of drops given by a stalagmometer of Traube, and calculated as mentioned in my former paper (this *Journal*, 1913, p. 316).

Table showing influence of Berkefeld filtration upon S.T.

Dist. water	100·0
M-piece diluted 1 : 10 with 0·85 % NaCl	102·16
„ „ „ „	filtered through Berkefeld filter	100·17
E-piece				Untreated	Filtered through Berkef.
diluted 1 : 4, not isotonic	104·30	102·41
„ 1 : 8, „	102·77	100·76
„ 1 : 10, isotonic	104·95	102·51
Serum diluted 1 : 10 with				0·85 % NaCl	Aqu. dist.
„ untreated	103·67	101·57
„ heated $\frac{1}{2}$ hour at 56° C.	106·87	106·38
„ filtered through Berkefeld filter	103·06	100·56

The table shows clearly that substances which lower the surface tension are kept back by filtration, thus producing a higher surface tension of the filtrate.

There are however other substances in the serum which lower the surface tension. Apart from the haemoglobin which according to Iscovesco (1911) lowers the surface tension of water, but the presence of which in the serum can be avoided, there are the pigments and the

lipoids of the serum as well as salts of fatty and gallic acids (J. Traube, 1908). The usual method of preparing a serum albumin by means of salting out with the sulphate of ammonium involves the pigments and the lipoids, the pigments coming down mainly with the albumin. It is therefore a matter of doubt whether the relatively large decrease of surface tension produced by the serum albumins is not at least partly due to the accidental presence of these substances, the more so as Iscovesco (1910) found that pure egg albumin even increases the surface tension of water. It would be of interest if egg- and serum-albumin are really different in their effect upon surface tension. That these serum pigments are readily adsorbed follows from the observation that the first parts of the filtrate are colourless; the serum colour reappears with further filtration.

As far as my experience is concerned, I agree with the statement of Bottazzi, that the serum albumins lower the surface tension of water (H. Schmidt, 1914). The albumins therefore tend to become adsorbed. If the serum has previously been heated, it has been shown by Traube, M. J. Gramenitzky, L. Berczeller, H. Schmidt and others, that a well-marked decrease of surface tension occurs. According to L. Berczeller (1913) this loss of surface tension is due to the production of albumoses and peptone.

From these observations it is easy to understand that a preliminary filtration of a thermo-inactivated serum (Muir and Browning) or of peptone (Holderer, 1912) facilitates the filtration of serum. Adsorption of the albumins diminishes their protective influence upon the dispersity and the solubility of the euglobulins. The dispersity especially of the euglobulins is decreased and as a consequence their filtrability is also decreased. But this may not be the only cause of the retention of the euglobulins. According to recent work of H. Chick (1913) the globulins form soluble compounds with the salt (NaCl) which are electrically neutral. As there is adsorption of the ions of the dissociated salts in the serum during filtration, as will be explained later, the globulin salt compounds tend to dissociate and become electrically charged, which renders them liable to anomalous adsorption. Quantitatively the amount of euglobulin in the serum is comparatively very small, and therefore its partial removal alters the mass relations of the proteins far more than a removal of a corresponding part of the albumins or the pseudoglobulins. This may help to explain the E-piece action of a Berkefeld filtrate.

In order to explain the influence of salts upon filtration through

a Berkefeld filter I would like to draw attention to a phenomenon, first described many years ago, but so far as I am aware not yet taken into account in the filtration of serum.

Quincke (1861) observed that in passing a liquid through a capillary tube a potential difference occurs between the capillary wall and the liquid. Helmholtz (1879) found an expression for the electromotive force thus developed according to which it is, apart from other relations, directly proportional to the hydrostatic pressure and inversely proportional to the viscosity and the electric conductivity of the liquid in question.

This has been confirmed later by J. Perrin (1904, 1905, 1906). Fr. Fichter (1911) and N. Sahlbohm (1910) drew attention to this phenomenon in explaining the precipitation which colloids with electro-positively charged particles undergo, when passing through capillaries, the diameter of which does not exceed 0.15 mm. N. Sahlbohm demonstrated this phenomenon to be a consequence of the movement of the liquid through the capillary and to be independent of the nature of the material of the capillary walls. Where there is no movement only adsorption takes place. The movement of the liquid produces a negative charge of the wall against the water, while in the case of electro-positively charged colloids, the colloidal particles are positively charged against the water as their dispersion medium. If the potential difference thus produced be large enough to overcome the positive charge of the particles the latter lose their charge and owing to surface tension aggregate and precipitate.

Now the unsalted E-piece obtained by dilution with water and acidifying with CO_2 has a distinct acid reaction and the protein particles in it are therefore positively charged (W. Pauli). It seems to me very probable that such an E-piece being an electro-positive colloid is to a large extent precipitated in the small capillaries of the filter and therefore not able to pass through a Berkefeld filter (cf. Exp. 5).

On the other hand, it is possible to assume that negatively charged protein particles will show a tendency to be repulsed by the negative charge of the Kieselguhr surface. This occurrence would inhibit the adsorption process and facilitate the passage. Positively charged particles would however show a tendency to adsorption, and the greater this potential difference the greater would be this tendency, but in the course of adsorption the potential difference is decreasing owing to the neutralising effect of the adsorbed particles and with that the adsorption decreases as well, till finally no more adsorption takes place.

The presence of electrolytes such as NaCl influences the phenomenon in the following manner: the NaCl is dissociated; its kation tends to neutralise the electric charge of the filter surface. This causes a decrease of the potential difference and therefore less repulsion of negative particles and less attraction or adsorption of positive particles. The influence of NaCl consists therefore in facilitating the adsorption of negatively charged particles and in diminishing the adsorption of positive particles. This is in analogy with the anomalous adsorption of dyes on filter paper described by W. M. Bayliss (1906) and may explain the results of Exp. 5.

It must however also be remembered that the addition of salts decreases a little the viscosity of proteins (W. Pauli), and increases the electric conductivity to a large extent. This latter factor will therefore, according to Helmholtz's formula, diminish the electromotive force produced by the movement in the capillaries, and in this way salts tend to prevent the precipitation of acid proteins. In addition to this the presence of salts (NaCl) decreases the degree of acidity and therefore the positive charge of the particles in an acid protein solution (H. Chick and C. J. Martin, 1913).

Finally I would like to draw attention to some experiments made by Holderer because these experiments, in spite of being made with Chamberland candles, verify to some extent the above described conceptions.

M. Holderer (1909, 1910) and M. Holderer and G. Bertrand (1910) found that many ferments which do not pass a Chamberland filter if in a medium neutral to methylorange ($H' = 10^{-4.4}$) will promptly do so if the acidity is reduced to the neutral point of phenolphthalein ($H' = 10^{-8.3}$). By the addition of neutral salts however Holderer (1910) succeeded in filtering the ferments even if the medium was only neutral to methylorange.

SUMMARY.

In the Berkefeld filtration of a fresh guinea-pig serum, adsorption on the filter surface takes place, and the serum passes unaltered, if the saturation of the adsorbing surface has become complete.

This adsorption involves most probably first the albumins, which process then secondly influences the filtrability of the globulins. It is also shown that the physical conditions of the serum as regards salt concentration and dilution as well as H' -concentration modify the effect of filtration to a large extent.

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THE INFLUENCE OF THE AGE OF THE PARENT ON THE VITALITY OF THE CHILD—A FIRST STUDY.

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THE subject I propose to discuss in the present and subsequent papers is of importance in two ways. Popular traditions have frequently implied that the age at which a parent begets offspring is not without influence upon the characters of the latter, the prevailing belief being humorously portrayed in the late W. S. Gilbert's ballad "The Precocious Baby." Again, recent thinkers, notably Professor Karl Pearson, have asserted that elder born children differ markedly in liability to certain diseases from their younger brethren. Were this view to be accepted it would be relevant to enquire whether the difference might not be a function of the age of the parents at the time of birth of the offspring or at least whether such difference might not play a part in bringing about this result. Assuming that any such effect could be demonstrated, it is plain that a valuable stimulus would be given to the study of physiological changes in the reproductive system within the fertile period. These are the immediately interesting aspects of the subject, but with them I am less directly concerned than with certain secondary consequences.

To state that an essential preliminary of a comparison is that the things to be compared must be in *pari materia*, may seem unnecessary, but this truism is in constant danger of being forgotten. We are all aware that the crude birth or death rates of different communities cannot be made the subjects of a valid comparison, but that corrections for age and sex distribution are necessary. The introduction of these

corrections has, however, tended to engender a feeling of undue confidence in the ease of interpreting such adjusted ratios. Quite recently warnings have emanated from various quarters with regard to this point. Thus Brownlee has pointed out in this *Journal* that the customary employment of a corrected death rate as a measure of salubrity is not free from doubt, while Hamer in his presidential address to the epidemiological section of the Royal Society of Medicine and again in his annual report to the London County Council has dwelt upon the possible importance of migration as a factor in death and birth rates which cannot be entirely allowed for by means of age and sex corrections. Evidently if the age at which a child is begotten or conceived, influences not merely the probable number of its brothers and sisters but also its own physical or mental characters, we have to reckon with another factor in the interpretation of a changing death or disease rate. It will not be sufficient to allow for the age constitution of a community, it will be necessary to consider that of the previous generation as well, since, whatever else may be obscure, it is certain that a small but significant increase in the average age at marriage has taken place in most sections of the community. For these reasons, it is plain that an attempt to measure the influence of the ages of parents at the time of procreation or conception upon the longevity of their offspring is worth making, and this paper contains an account of a preliminary effort. I shall not try to summarise the literature, at once voluminous and meagre, which has some more or less direct bearing upon my theme; much of it is merely traditional and theoretic, little of it is based upon exact statistical data handled in an appropriate fashion. Stanley Hall's well-known book on adolescence contains references to various earlier papers, and additional citations will be found in Gini's 1912 paper, while among writers who have treated of the influence of order of birth, mention should be made of Lucien March, Karl Pearson and his associates, Weinberg, Yule and Greenwood, Goring and Ploetz. I may also refer to some preliminary notes of my own, cited in the list of references appended. This paper is confined to a study of the influence of parental age upon the longevity of offspring and age at marriage, and the data used will in the first place need careful description. These data fall into four series, viz. :

The first was collected from Burke's *Peerage*, 1902, and related to the possible influence of the age of the father at birth, on the age at death of sons who had survived to the 15th year; the first male born of the first male in each generation only being considered.

The second was collected in Middlesbrough, through the agency of the Medical Inspection of School Children, and deals with the age of the grandmother at the birth of the mother and the number of children dying before adult life.

The third also collected in Middlesbrough, through the agency of the Notification of Births Act, permitted the consideration of the age of mother at birth of the offspring and its chance of living one year.

The fourth and last collected through the same agency as the third, had for its object the possible association between age and the chance of foetal death prior to term.

It will be observed, that, though there is some overlapping, all periods of life are covered and further that both parents have been dealt with. Unfortunately data were not obtainable in the *Peerage* with respect to the age of the mother. It is obvious that in an enquiry of this kind, both parents ought to be considered, for should an association be found to exist in the case of the male parent, it might arise from the ages of the uniting ovum and sperm or the environmental influence of the mother's age, subsequent to fertilization. We can now consider each series in detail, indicating the corrections that should be made and such fallacies as are dependent upon the method of collecting the data.

Age of Father at Son's Birth, and Age at Death of Adult Sons.

The peculiarities of this series of observations which should be borne in mind are as follows :

(a) The oldest males only were considered. That is, only instances where the father was the eldest born and the son whose age at death was recorded was also the first male born. Unfortunately the actual position in the family of the person under consideration is not given in the raw material.

(b) Only such sons as survived the 15th year are considered.

(c) All deaths through violence were excluded so far as recorded.

It has already been shown by Beeton, Pearson and Yule, that a correlation exists between the length of life of the father and that of the son which is equal to $\cdot 12$. Now it is obvious that a child born of an elderly parent may be influenced in two ways: (1) He may tend to survive to a mature age because his father has already done so and (2) his life expectancy may be curtailed because his parents were

TABLE III.
Age of father at birth of eldest son and death of father.

		Age at death																				Totals														
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	5	6													
20	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1													
22	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1													
24	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1													
26	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1													
28	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1													
30	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1													
32	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1													
34	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1													
36	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1													
38	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1													
40	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1													
42	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1													
44	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1													
46	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1													
48	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1													
50	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1													
52	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1													
54	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1													
56	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1													
58	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1													
60	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1													
62	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1													
64	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1													
66	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1													
68	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1													
70	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1													
72	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1													
74	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1													
76	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1													
78	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1													
80	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1													
Totals		5	3	4	10	11	27	11	15	33	43	42	38	27	48	41	41	59	49	58	70	69	61	70	85	67	43	40	83	23	7	11	4	1	1	1201

$\sigma_x = 4.1604$, $\sigma_y = 7.3045$, $r = + .2543 \pm .0229$.

The values of the correlation disclosed by the tables are as follows :

Age of father at birth (1) and son at death (2), $r = .0345 \pm .023$

Age of father at death (3) and son at death (2), $r = .2248 \pm .0248$

Age of father at birth of son (1) and death (3), $r = .2543 \pm .0229$

It is to be observed that the second coefficient is much larger than that previously given by Prof. Pearson and Miss Beeton, namely $.135 \pm .0209$, and the matter needs further discussion. In the first place the correlation surface is not a random sample of the type descriptive of the degree of association obtaining in the whole "population" of fathers and sons owing to the restriction as to age and birth order already mentioned. So far as the former is concerned the same selection was practised by Beeton and Pearson in their paper of 1899 (*Proceedings, Royal Society*) which also dealt with peerage data, and the correlations they obtained are decidedly smaller than mine; but they did not restrict the analysis to first male births. This may partly account for the discrepancy between our results. It will be noticed that there is far less difference in mean age and variability of father and sons in my data than in that of Beeton and Pearson. It will be remembered that the latter pointed out (*op. cit.* p. 292, etc.) that the filial generation was less stringently selected than the parental. The observed correlation was necessarily lowered, there being a mixture of material due to the operation of that fraction of the death rate which is really non-selective upon the sons. It appears probable that the further selection in confining oneself not only to adult firstborn males, but to those who actually survived their fathers, is a sufficient explanation of the difference. Whether my value is a really adequate measure of the force of inheritance in respect of longevity and what part is played in heredity by the influence of the age of the parents at birth of the son cannot be stated off-hand.

In the following table (p. 460) are the constants as given by Beeton and Pearson in their 1899 paper and found from the above data.

If we now make age of father at death constant then

$$r_{12} = - .0431 \pm .0239.$$

The conclusion or rather suggestion is that as the father increases in age at the birth of the son, the life expectancy of the son, who has already survived to the 16th year, tends to be curtailed. The correlation ratio for means of arrays of sons' ages at death for various ages of fathers at birth of sons = $.3394 \pm .0389$, and when corrected by Pearson's method (*Biometrika*, VIII) the value approximates to .3042.

	Beeton and Pearson's data (1899)	Present Paper
Mean age of Fathers at death* ..	65.835	67.14
Mean age of Sons at death ..	58.775	65.84
Standard Deviation (Fathers) ..	14.6382	14.24
Standard Deviation (Sons) ..	17.0872	14.20
Coefficient of correlation	0.1149 \pm .0210	.2214 \pm .0248
Correlation ratio (Fathers)† ..	—	.3394 \pm .0389
Corrected‡	—	.3042

* The mean age of fathers who were Peers is one and a half years greater than the sons who succeeded them. This is due to the fact that a proportion did not inherit the title and hence would be of a mature age when it was conferred upon them.

† Any distribution has two correlation ratios; the word in brackets indicates which has been calculated, *e.g.* correlation ratio (fathers) denotes the coefficient obtained from the means of arrays of fathers.

‡ The correlation for η is calculated by the method described by Prof. Pearson, 1912.

If we replaced number of years lived by the death rate at ages and thus reversed the order of our categories, the association would become positive and we might say that the death rate at ages increases with the age of the father at birth. This change as will be seen subsequently would bring the present series of observations into line with the remainder.

The Age of the Grandmother at the Birth of the Mother and the Number of Children dying in the Family.

This enquiry as already stated was carried out in Middlesbrough through the agency of the Education Act (Administrative Provisions) 1907, or what is known as the Medical Inspection of School Children. The method was as follows: All children in their 8th year were selected and their parents invited to attend the examination; of the total number, 50% complied. The sample thus obtained can be regarded as comprehensive and is such that it reaches each family once only. It is obvious, however, that the material is a selection and not a random sample because it ignores all sterile matings and those in which all the children born have died before the 8th year; further the representation of families of various sizes is directly proportional to the number that survive. Hence the larger the family the greater the chance of its possessing a member 8 years old.

According to the work of Weinberg, Macaulay, Yule and Greenwood it appears that considerable caution must be exercised in basing deductions on data collected in this way, as the possible statistical fallacies are numerous and in some respects cannot be corrected.

One error which tells against a positive conclusion arises from the fact that if age at birth is detrimental to the survival value of the family, it is obvious that those families which have been hit the hardest are only represented in our data in proportion to the chance of one of the survivors being 8 years old, which, even if the family was originally large, might be small, under the method of selection adopted.

It is to be regretted that no allowance has been made for the length of time the mother had been married, as the age at marriage was not obtained in the original data. To assume that the mean reproductive periods in the categories chosen will be approximately equal is an assumption that is hardly justifiable. Bearing these reservations in mind we may proceed to discuss the actual findings. The information obtained from each mother or responsible guardian was (1) present age of mother; (2) age of grandmother if living, or age she would have been had she lived; (3) the number of children born to mothers—still births are included, but not miscarriages; (4) the number dead.

It is to be noted that information relative to ages is fairly reliable, owing to the widespread use of insurance against burial expenses at death. As in the previous case, partial correlation was used and the problem was to find the value of the association, if any, between the age at which the mother was born and the number dying for total number born constant.

The correlations for the above tables are as follows:

Age of grandmother at birth of mother (1) and number dead (2):

$$r_{12} = \cdot 0238 \pm \cdot 0213.$$

Number of children dead (2) and size of family (3):

$$r_{23} = \cdot 7230 \pm \cdot 0109.$$

Size of family born (3) and age of grandmother at birth of mother (1):

$$r_{13} = \cdot 0641 \pm \cdot 0150.$$

Testing our crude correlations for linearity, we have for age of grandmother at birth of mother and number dying:

$$\eta_{12} \text{ (number dead)} = \cdot 076.$$

Corrected $\eta_{12} = \cdot 0314 \pm \cdot 0209,$

$$\eta_{13} \text{ (age)} = \cdot 139.$$

Corrected $\eta_{13} = \cdot 1015 \pm \cdot 0208.$

There may be some non-linearity with respect to the age categories, still a partial correlation should give a little information.

TABLE IV.
Age of grandmother at birth of mother and number of children dead.

Age at birth	0	1	2	3	4	5	6	7	8	9	10	11	12	No. dying, Means
20 yrs. and under ..	38	24	30	6	6	5	4	1	1		1			116
21st to 25th year..	95	68	45	23	19	5	3	2	1					1-724
26th to 30th year..	95	75	49	25	11	6	3	2	1					262
31st to 35th year..	78	46	42	15	16	6	5	4	2	2		1	1	273
36th to 40th year..	31	32	19	15	8	3	1	3	1		1			216
41st and over	22	13	11	7	1	3	1	1						113
Totals	359	258	196	91	61	28	17	13	6	2	5	2	1	1039
Means	3-09	3-12	3-04	3-34	3-06	3-21	3-52	3-69						1-621

$\sigma_x = 1.351, \sigma_y = 1.8486, r = .0238 \pm .0213.$

TABLE V.
Age of grandmother at birth of mother and number of children born to mother.

Age at birth	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	No. of children born
20 yrs. and under ..	6	16	19	14	22	15	11	9	4	3	5	1		3	1		129
21st to 25th year ..	14	28	65	57	43	28	17	21	26	9	4	1		3		1	320
26th to 30th year ..	17	49	50	43	53	34	17	16	14	10	5	3	2	5			318
31st to 35th year ..	17	42	46	45	48	24	13	14	8	7	4	3	1	1			273
36th to 40th year ..	15	25	40	18	16	11	10	10	6	3	4	1	1		2		162
41st and over	1	17	19	16	4	9	7	7	2	2	3	1	2	1			91
Totals	70	177	239	193	186	121	75	77	60	34	25	10	9	13	3	1	1293
Means	3-34	3-47	3-33	3-23	3-02	3-15	3-20	3-21	2-9	3-12	3-28	3-5	3-54	2-61	3-68		5-77

$\sigma_x = 1.473, \sigma_y = 2.8561, r = -.064 \pm .015.$

TABLE VI.

Size of family and number dead.

No. dead	Size of family																			Totals
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	
0	31	73	71	82	49	27	16	7	4	4	1									365
1		12	44	51	62	36	15	21	14	4	0	1	1							261
2			14	20	27	38	30	40	11	11	3	1	1	1						197
3				1	10	17	13	18	17	10	7	2	0	2						97
4					1	6	6	10	10	13	8	1	1	3	2					61
5							5	2	1	1	4	6	5	2						26
6							1	3	2	3	1	4	1	1		1				17
7									2	1	2	2	2	1	1					11
8										1	0	1	1	1	1				1	6
9											1	0	0	1						2
10														3		1				4
11														1						1
12														1					1	2
Totals ..	31	85	129	154	149	124	86	101	61	48	27	18	12	17	4	2	0	0	2	1050

$\sigma_x = 3.122$. $\sigma_y = 1.84$. $r = .723 \pm .0109$. Mean size of family 5.77. Mean number dead 1.56

Age of grandmother at birth of mother and number dying in the family, total number constant:

$${}_3r_{12} = .0943 \pm .0174.$$

This would suggest that as the age at which the mother was born increases, the chance of her rearing all her children decreases. It is of interest to note that the association having regard to its probable error is more significant than in our first series. Had the present data been as reliable we should have been justified in assuming that the effect of parental age at birth is more marked in early life than at the more mature ages.

The Age of the Mother at Birth of Child and its chance of living one year.

The third series of observations dealing with the possible association between age of mother at birth and chance of living one year is also subject to certain reservations:

- (1) The data deal only with a poor class population.
- (2) Only such as remain in the town for one year are considered.
- (3) A fair number are lost owing to migration. These are usually the latter born, for it is the parents of such who are most affected by

trade fluctuations. The number lost in this way is a little over 5% of the whole number. The information collected was :

(1) Age of mother at birth.

(2) Order of birth. (Miscarriages and still births are included.)

(3) Whether living or dead at end of the year. This information is of course furnished by the Registrar to all Health Departments. The method adopted was as follows: A table was formed (Table VII) with age of mother at birth and order of birth as abscissae and ordinates respectively, and in each appropriate square the number born and the number dead at the end of the year were placed and the death rate for each age and order worked out. This table was then turned into two sub-tables :

(1) Infantile Mortality and age of mother at birth.

(2) Order of birth and Infantile Mortality.

The figures in the squares being the number of observations upon which the various mortality categories are based. The third table, Age of mother at birth and order of birth, was taken from the original table.

The constants obtained from these tables are :

Age of mother at birth of offspring (1) and order of birth (3) :

$$r_{13} = \cdot 6128 \pm \cdot 0051.$$

Age of mother at birth of offspring (1) and infantile mortality (2) :

$$r_{12} = \cdot 1157 \pm \cdot 0142.$$

Order of birth (3) and infantile mortality (2) :

$$r_{23} = + \cdot 0367 \pm \cdot 0208.$$

Taking the age of mother at birth of offspring and infantile mortality, with order constant :

$${}_3r_{12} = \cdot 1179 \pm \cdot 0143,$$

and birth sequence and infantile mortality with age of mother at birth constant :

$${}_1r'_{23} = - \cdot 0531 \pm \cdot 0205.$$

Testing these for linearity of regression, order of birth and infantile mortality

$$\eta \text{ (order)} = \cdot 2403, \eta \text{ (corrected)} = \cdot 2254 \pm \cdot 0145.$$

Age of mother and infantile mortality :

$$\eta \text{ (mortality)} = \cdot 2939, \eta \text{ (corrected)} = \cdot 2810.$$

TABLE VII. *Number of births and deaths in first year according to age of mother at birth and order.*

Years	1st	2nd	3rd	4th	5th	6th	7th	8th	9th	10th	11th	12th	13th	14th	15th	16th	17th	No. born	Deaths	Rate per 1000
	1																	1	—	0
15	—	2																2	—	0
16	—	7																7	2	286
17	2	2	1															20	2	100
18	2	—	—															49	14	286
19	35	14																75	10	133
20	5	9																78	14	179
21	61	11	2	1														94	15	160
22	7	2	1	—														121	17	140
23	49	20	6	1	2													106	9	83
24	10	3	—	—	1													145	17	117
25	45	29	14	6														133	18	135
26	7	4	4	—														98	15	153
27	39	41	28	8	5													117	13	111
28	6	7	4	—	—													111	15	104
29	29	31	22	11	9	3	1											107	14	131
30	1	4	3	—	1	—	—											76	13	171
31	45	27	36	26	7	4												107	15	140
32	6	4	5	1	1	—												88	16	182
33	29	37	29	23	11	4												107	11	103
34	7	4	2	4	1													85	12	141
35	17	18	19	15	15	10	1	1	1									72	9	125
36	2	3	—	2	4	2	—	—	—									58	10	172
37	16	23	27	20	18	4	6	3										65	11	169
38	2	5	1	3	1	—	—											49	6	122
39	15	10	23	19	16	16	9	2	1									57	13	232
40	4	—	5	2	—	—	2	2	—									35	8	229
41	4	13	12	20	23	18	10	6		1								24	3	125
42	2	2	1	3	1	1	1	2		1								27	6	222
43	2	8	10	8	10	13	18	5	1	1								18	3	167
44	1	—	2	1	2	—	4	2	—	1								17	5	294
45	3	8	8	14	16	24	11	13	5	5								6	0	0
46	—	—	—	2	1	2	5	1	1	3								1	0	0
47	6	3	4	7	8	21	20	9	4	2	3	1						2	0	0
48	2	—	—	2	1	5	3	1	1	—	1	—	1	1				1	0	0
49	3	4	6	8	11	18	17	17	5	8	4	3	1	—	—			1	0	0

No. of births: 437 313 269 212 195 187 149 114 82 72 55 31 14 14 7 4 4 2159

No. of deaths:

67 54 32 23 22 16 20 24 12 18 8 8 4 3 4 1 0

Rate .. 146

The regression, therefore, is markedly non-planar. This is probably due to the fact that the data are heaped up in the two extreme categories, namely, mortality of $\cdot 0-5\%$, the largest portion consisting of families in which none have died in the first year and mortalities of $90-100\%$, mainly those families in which all have died (a fact which in the majority of cases was due to syphilis). In spite of these reservations, I think we can conclude that as the age of the mother advances, the chance of the child surviving decreases, and as the family gets larger, other things being constant, the probability of its living one year increases. Findings which are on the whole corroborated by one's general impressions. Since the third series is restricted to infantile mortality which is only a part in the second series, the suggestion is that the influence of parental age hardly affects the adolescent period of life. It must, however, be remembered that the methods of tabulation were not the same in the two cases.

Present Age of Mother and Number of Accidents in previous Family.

The fourth and last series was obtained with the object of ascertaining the possible association between the age of the mother and the death of the foetus prior to full gestation; the data were collected in Middlesbrough on the occasion of a birth. The mother being asked (1) the number of live births she had had; (2) the number of deaths in the first year; (3) the number of miscarriages and still births. It is to be noted first, that the enquiry was limited to certain working class districts of the town, though all births were reached that occurred in that area, and secondly, that each category is assumed to include the experience of the previous ones. This is not quite true, as the smaller families tend to be restricted to ages centring round the 29th year and are more rarely found in the early and late periods. The data are on these accounts obviously not homogeneous. The findings, however, in the previous series that size of family does not seriously affect infantile mortality when age is made constant, should remove this objection.

The data are as follows:

TABLE VIII.

*Histories of mothers with respect to accidents, deaths in first year,
and number of births in first year.*

Age of mother	No. of enquiries	No. of births	Accidents	Rate	No. of deaths	Rate
16	4	4	0	0	0	0
17	10	10	0	0	0	0
18	26	32	6	188	2	63
19	51	63	1	16	6	95
20	82	111	3	27	9	81
21	89	154	12	78	14	26
22	102	218	19	87	16	73
23	129	313	19	61	31	99
24	118	321	24	75	39	121
25	110	321	18	56	37	115
26	110	384	31	81	48	125
27	100	399	36	90	66	165
28	103	421	26	62	66	157
29	98	463	27	58	59	127
30	101	426	41	96	75	176
31	68	307	16	52	31	101
32	88	522	36	69	61	117
33	75	479	32	67	66	138
34	77	559	68	122	69	123
35	61	437	26	60	44	111
36	63	413	25	61	59	143
37	54	425	33	78	68	160
38	44	396	47	119	45	114
39	40	381	61	158	62	163
40	41	393	34	87	60	153
41	9	110	8	73	17	155
42	23	289	44	152	44	152
43	12	138	13	94	12	86
44	12	128	12	94	30	234
45	3	42	4	95	1	24
46	3	30	4	133	1	33
47	1	9	0	0	2	222
48	1	13	0	0	1	77
49	1	12	0	0	2	106
Totals	1909	8728	726	86	1143	119

Correlating these directly and using the column "Number of Enquiries" as weights, we have:

Present age of mother and infantile mortality in previous family :

$$r = .0425 \pm .0210 ;$$

present age of mother and accident rate in previous family :

$$r = \cdot 2886 \pm \cdot 018 ;$$

infantile mortality rate and accident rate :

$$r = \cdot 4006 \pm \cdot 0064.$$

Considering the problem from the point of view of individual families we see that as the age of the mother increases so does the chance of the child dying either before or after birth increase, the correlation in the former case being about seven times that in the latter. It is of interest also to note the high correlation between death before and after birth.

Putting the foregoing results together we have,

- (1) Present age of mother and miscarriage rate in previous family :

$$r = \cdot 2886 \pm \cdot 018 ;$$

- (2) Age of mother at birth of offspring and infant mortality :

$$r = \cdot 1179 \pm \cdot 0143 ;$$

- (3) Age of grandmother at birth of mother and number of family dying before adult life :

$$r = \cdot 0943 \pm \cdot 0174 ;$$

- (4) Age of father at birth of son and longevity of sons :

$$r = - \cdot 0431 \pm \cdot 0239.$$

If, however, we could exclude from (3) the association due to infantile mortality, it might approximate to zero or actually become negative. It is possible that we are dealing with two different things, namely, sthenic and asthenic deaths. In the former the reactions of the organism are so excessive as to cause death, whilst in the latter the death is due to their relative absence. In infancy and late life death is mainly asthenic, whilst in the adolescent period it is sthenic. If this is so, then a negative correlation for the adolescent period would be expected. The above consideration, though it goes beyond the data, is a useful suggestion as it indicates that the next step in the enquiry must be the consideration of age of parent at birth of offspring and cause of death, age at death being kept constant.

Age of Father at birth of Son and Age of Son at Marriage.

As evidence has already been given which lends considerable force to the belief that the number of years that a person will live partly depends upon the age of the parents, when he or she was born, it

is not unreasonable to consider whether fertility is also influenced by the same factor. Before such an enquiry can be undertaken, the factors limiting the reproductive period must be clearly defined. The termination, that is death, has already been dealt with in the present paper, and the beginning, namely age at marriage, will now be examined.

The data on which this investigation is based have been taken from Burke's *Peerage*, and to produce as much homogeneity as possible only eldest born sons of eldest born fathers are considered. The characters chosen were :

- (1) Age of father at birth of his son.
- (2) Age of son at marriage.
- (3) Age of father at death.
- (4) Age of father at marriage.

Beyond the two fundamental factors it is necessary to allow for any hereditary tendency, first in respect to marriage, for if the father mates early the son is likely to do so, and secondly in respect to death, for if the father dies early it is likely to hasten the marriage of the son, as he will assume his full authority at an earlier date, and should he be a minor at the time there will be every social reason for his marriage at the earliest moment that convention allows, hence it is necessary to examine any association that may exist between the factors: Age of the father at marriage and age of son at marriage, Age of father at death and age of son at marriage, and should such prove to exist, suitable allowance must be made. The values are as follows :

Age of father at birth of son (1) and age of son at marriage (2) :

$$r_{12} = -\cdot0175 \pm \cdot0247.$$

Age of father at birth of son (1) and age of father at marriage (4) :

$$r_{14} = \cdot7338 \pm \cdot0114.$$

Age of father at birth of son (1) and age of father at death (3) :

$$r_{13} = \cdot2534 \pm \cdot0232.$$

Age of father at marriage (4) and age of father at death (3) :

$$r_{34} = \cdot1620 \pm \cdot0239.$$

Age of son at marriage (2) and age of father at death (3) :

$$r_{23} = \cdot2219 \pm \cdot0236.$$

Age of son at marriage (2) and age of father at marriage (4) :

$$r_{24} = \cdot0463 \pm \cdot0243.$$

TABLE IX.
Age of father at birth of son and age of son at marriage.

Age of father at birth		Age of son at marriage																												Totals	
15-16	17-18	19-20	21-22	23-24	25-26	27-28	29-30	31-32	33-34	35-36	37-38	39-40	41-42	43-44	45-46	47-48	49-50	51-52	53-54	55-56	57-58	59-60	61-62	63-64	65-66	69-70	71-72	77-78	Totals		
1	1	1	1	1	3	1	2	2	1	2	2	2	1	2	3	1	1	1	1	1	1	1	1	1	2	1	1	1	1	3	
1	1	1	5	7	10	15	14	18	11	12	5	6	5	3	3	2	2	2	5	1	1	1	1	1	1	1	1	1	1	4	
1	1	1	12	19	17	17	11	13	11	7	4	10	9	3	3	2	1	1	1	1	1	1	1	1	1	1	1	1	1	26	
1	1	1	8	5	11	14	13	21	16	6	11	8	7	3	3	2	2	3	1	1	2	1	1	1	1	1	1	1	1	137	
1	1	1	5	11	13	11	14	9	7	13	7	4	6	1	2	5	2	3	1	1	2	1	1	1	1	1	1	1	1	110	
1	1	1	3	7	5	8	6	12	6	10	7	7	4	3	3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	88	
1	1	1	1	2	4	6	3	10	5	6	3	4	3	3	3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	64	
1	1	1	1	3	3	5	6	4	4	5	2	2	3	1	1	2	2	1	1	1	1	1	1	1	1	1	1	1	1	63	
1	1	1	1	3	3	5	4	3	2	5	3	2	3	1	1	2	2	1	1	1	1	1	1	1	1	1	1	1	1	43	
1	1	1	1	4	4	6	4	4	5	2	4	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	35	
1	1	1	1	3	2	2	2	1	1	2	3	2	1	1	1	2	2	1	1	1	1	1	1	1	1	1	1	1	1	21	
1	1	1	2	1	1	1	2	2	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	20	
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	16	
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	7	
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	5	
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	4	
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	3	
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1								

$\sigma_{\text{birth}} = 3.7673$, $\sigma_{\text{marriage}} = 4.3389$, $r = -.0175 \pm .0247$.

TABLE X.
Age of father at marriage and age at birth of eldest son.

Birth of eldest son	Age at marriage																							Totals
	13-14	15-16	17-18	19-20	21-22	23-24	25-26	27-28	29-30	31-32	33-34	35-36	37-38	39-40	41-42	43-44	45-46	47-48	49-50	53-54	55-56	57-58	59-60	
15-16	1																							1
17-18		2	2																					2
19-20			2	1	18																			4
21-22			2	1	12	17																		31
23-24			2	1	37	24	13																	57
25-26		1		3	36	22	40	22																71
27-28			1	2	8	6	11	11	19															92
29-30				1	5	11	11	22	34	11														71
31-32				1	2	6	11	10	8	13	9													91
33-34				1	2	2	2	3	7	8	18													63
35-36				1	2	1	3	3	2	6	6	9												59
37-38				1	2	1	3	2	2	3	4	5	14	2										44
39-40				1	3	5	1	1	1	2	3	5	5	3	3									35
41-42					3	5	2	2	1	1	1	2	5	3	3	3								31
43-44					2	1	2	2	1	1	1	2	5	2	2	2								23
45-46				1			2	1	4	1	1	1	1	1	1	1	3							19
47-48								1			1	1	1	2	1	4		1						11
49-50									1		1	1		2	1		2	1	3					6
51-52																								5
53-54					1	1				1		1			1									2
55-56																1								3
57-58											1			1						1	1			4
59-60																				1				1
61-62																								1
63-64																								1
65-66																								2
67-68																								1
69-70				1															1					3
71-72																								1
Totals	1	3	8	30	115	104	99	92	76	47	44	35	31	17	13	10	5	2	5	2	1	5	1	746

$\sigma_{\text{marriage}} = 3.5564, r = .7338 \pm .0114.$
 $\sigma_{\text{age at birth}} = 4.3112.$

$\sigma_{\text{age at birth}} = 4.3112$, $\sigma_{\text{marriage}} = 3.5564$, $r = .7338 \pm .0114$.

TABLE XI.
Age of father at marriage and age of father at death.

Age at death		Age at marriage																				Totals
23-24	25-26	27-28	29-30	31-32	33-34	35-36	37-38	39-40	41-42	43-44	45-46	47-48	49-50	53-54	55-56	57-58	59-60	Totals				
1	1	1	1	1	3	1	3	1	5	1	1	2	1	1	1	1	1	1				
1	1	1	1	3	1	1	2	1	5	1	1	1	2	1	1	1	1	1				
1	1	1	1	5	1	3	4	1	1	1	1	1	2	1	1	1	1	1				
1	1	1	1	3	1	1	2	1	1	1	1	1	2	1	1	1	1	1				
1	1	1	1	6	2	2	3	1	2	2	2	1	1	1	1	1	1	1				
1	1	1	1	10	8	6	3	4	1	3	1	1	1	1	1	1	1	1				
1	1	1	1	8	8	6	2	3	2	2	2	1	1	1	1	1	1	1				
1	1	1	1	12	11	5	6	3	3	3	2	1	1	1	1	1	1	1				
1	1	1	1	9	6	9	17	5	10	7	3	4	1	1	1	1	1	1				
1	1	1	1	13	17	13	5	10	3	5	5	3	3	1	1	1	1	1				
1	1	1	1	12	12	12	15	14	7	5	5	5	2	2	2	2	2	2				
1	1	1	1	11	13	5	13	7	3	3	3	3	2	1	1	1	1	1				
1	1	1	1	9	9	8	8	6	2	6	5	5	4	1	1	1	1	1				
1	1	1	1	6	8	2	2	9	3	3	3	3	2	1	1	1	1	1				
1	1	1	1	9	9	8	8	6	4	5	5	5	3	1	1	1	1	1				
1	1	1	1	1	1	2	1	1	2	2	2	2	1	1	1	1	1	1				
1	1	1	1	2	1	1	1	4	2	5	5	5	3	1	1	1	1	1				
1	1	1	1	2	1	1	1	2	1	1	1	1	1	1	1	1	1	1				
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1				
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1				
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1				
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1				
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1				
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1				
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1				
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1				
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1				
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1				
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1				
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1				
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1				
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1				
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1				
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1				
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1				
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1				
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1				
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1				
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1				
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1				
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1				
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1				
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1				
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1				
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1				
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1				
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1				
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1				
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1				
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1				
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1				
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1				
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1				
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1				
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1				
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1				
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1				
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1				
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1				
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1								

$\sigma_{\text{death}} = 3.5118$, $\sigma_{\text{marriage}} = 3.4230$, $r = .16198 \pm .0239$.

TABLE XII.
Age of son at marriage and age of father at death.

Age at death	Age at marriage																								Totals	
	13-14	15-16	17-18	19-20	21-22	23-24	25-26	27-28	29-30	31-32	33-34	35-36	37-38	39-40	41-42	43-44	45-46	47-48	49-50	51-52	53-54	55-56	57-58	63-64		71-72
25-28																										
29-32				1	1	1	1	1	1	1	1	3		1					1							
33-36				1	1	1	1	1	1	1	1															
37-40				1	1	1	1	1	1	1	1															
41-44				1	1	1	1	1	1	1	1															
45-48	1		1	3	2	3	5	4	4	4	1							1								
49-52				1	1	1	1	1	1	1	1															
53-56				1	1	1	1	1	1	1	1															
57-60				1	1	1	1	1	1	1	1															
61-64				1	1	1	1	1	1	1	1															
65-68				1	1	1	1	1	1	1	1															
69-72				1	1	1	1	1	1	1	1															
73-76				1	1	1	1	1	1	1	1															
77-80				1	1	1	1	1	1	1	1															
81-84				1	1	1	1	1	1	1	1															
85-88				1	1	1	1	1	1	1	1															
89-92				1	1	1	1	1	1	1	1															
93-96				1	1	1	1	1	1	1	1															
101-104				1	1	1	1	1	1	1	1															
109-112				1	1	1	1	1	1	1	1															
113-116				1	1	1	1	1	1	1	1															
Totals	1	2	3	19	94	115	104	84	76	60	46	27	32	16	7	11	5	5	6	3	2	1	1	2	1	723

$\sigma_{\text{death}} = 24.322$
 $\sigma_{\text{marriage}} = 3.5867$
 $r = .2219$
0.236

$\sigma_{\text{death}} = 3.4322, \quad \sigma_{\text{marriage}} = 3.5867 \quad r = .2219 \pm .0236.$

TABLE XIII.
Age at marriage of father and age at marriage of son.

		Son																											
		13-14	15-16	17-18	19-20	21-22	23-24	25-26	27-28	29-30	31-32	33-34	35-36	37-38	39-40	41-42	43-44	45-46	47-48	49-50	51-52	53-54	55-56	57-58	63-64	73-74	Totals		
Father		1	1	1	1	2	1	1	1	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1		
13-14	1																										1		
15-16		1																									1		
17-18			1																								1		
19-20				1																							1		
21-22					1																						1		
23-24						1																					1		
25-26							1																				1		
27-28								1																			1		
29-30									1																		1		
31-32										1																	1		
33-34											1																1		
35-36												1															1		
37-38													1														1		
39-40														1													1		
41-42															1												1		
43-44																1											1		
45-46																	1										1		
47-48																		1									1		
49-50																			1								1		
53-54																				1							1		
55-56																					1						1		
57-58																						1					1		
59-60																							1				1		
Totals	1	2	3	18	89	112	105	84	74	59	47	28	31	16	7	11	5	5	6	3	2	2	1	1	2	1	713		

$\sigma_{\text{father}}=3.4747$
 $\sigma_{\text{son}}=3.4683$
 $r = -.04627 \pm .0243$

$\sigma_{\text{father}} = 3.4747$, $\sigma_{\text{son}} = 3.4683$, $r = .04627 \pm .0243$.

TABLE XIV.
Age of father at marriage and age at death of eldest son.

Death of son	Marriage of father																Totals
	Under 20	21-24	25-28	29-32	33-36	37-40	41-44	45-48	49-52	53-56	57-60	61-64	65-68	69-72	73-76	77-80	
13-14																	4
15-16																	3
17-18																	6
19-20																	11
21-22																	15
23-24																	33
25-26																	33
27-28																	43
29-30																	47
31-32																	41
33-34																	47
35-36																	74
37-38																	70
39-40																	87
41-42																	62
43-44																	52
45-46																	34
47-48																	11
49-50																	2
51-52																	
53-54																	
55-56																	
57-58																	
59-60																	
Totals	1	3	8	30	115	103	99	92	75	47	34	32	18	13	10	5	746

$\sigma_{\text{marriage}} = 3.5429$, $\sigma_{\text{death}} = 3.7561$, $r = -0.298 \pm 0.242$.

Only two of these coefficients require note as the other four must necessarily be large owing to the nature of the data. r_{12} is negative, though small; as all the others are positive, it will be larger and still negative when the other variables are made constant. r_{24} is small but positive, this is due not to a transmitted tendency, but to the fact that the data cover a large period of time during which the marriage conventions have altered, and hence a slight positive association is produced. The restriction of the data as already narrated was not sufficiently stringent to remove it. Making the age of the father at death and marriage constant, we have:

$${}_{34}r_{12} = -\cdot1107 \pm \cdot0211.$$

It would thus appear that a negative association exists between the age of the father when the son is born and the age at which the latter marries. It must be admitted that the value obtained is open to doubt; it is questionable as to whether it is really necessary to make the age of the father at death constant in all cases, for it may be, that only in the instances in which the son is a minor at the father's death is correction needful. Still, if we suppose that the true value lies between $-\cdot1107$ and $-\cdot0175$, the total and partial values, it would still suggest that a negative association exists.

A seventh table has been added to the above series, namely, Age of father at marriage and age at death of eldest son; in this case $r = -\cdot0298 \pm \cdot0246$, this lends support to the value of the partial r found for Age of father at birth of son and age of son at death, namely $r = -\cdot0434 \pm \cdot0239$.

The following series of observations are of some interest if the data can be said to be free from fallacy.

The information was obtained from such parents as attended the examination of their children under the provisions of the Education Act of 1907. As the particulars under consideration were limited to one age period, each pair was recorded once only. It is to be noted, however, that when information is obtained through the medium of offspring respecting characters pertaining to the parents, it is subject to certain fallacies that have already been mentioned and it may be that the following results are dependent on the number of surviving children possessed by certain of the categories into which the data were divided, rather than a consequence of the existence of a relationship between the age of the parents at birth of mates. This association is to be expected if age at birth influences age at marriage, for if a man

mates early because his father was old when he was born, that man must possess qualities which differentiate him from others, and in so far as assortative mating is an accepted fact a positive correlation should exist between the ages of parents at birth of mates.

The point is one of interest and it would be advisable that this portion of the enquiry should be re-examined through the material directly obtained by the Registrars on the occasion of the registration of a marriage. The constants for these observations are :

Age of mother at birth of husband, and age of mother at birth of wife :

$$r = \cdot 01798 \pm \cdot 0194.$$

It may be doubted whether it is justifiable to regard age as a simple variable or to consider definite periods of time as representative of changes that occur in an organism, dependent upon duration ; that is to say, it is doubtful whether the period designated by the 50th year measures something similar to that of the 20th. If this is the case then a contingency table would give a better reflection of the bias if such exists, than the fitting of a straight line to a series of means.

The coefficient of contingency $C_2 = \cdot 1654$. If corrected

$$C_2 = \cdot 0964 \pm \cdot 0214$$

(approximate only, computed by $\frac{1 - C_2}{\sqrt{\eta}} \cdot 67449$).

If we evaluate the correlation ratio

$$\eta \text{ (husbands' mothers) } = \cdot 06496 \pm \cdot 0181.$$

The linear prediction formula is $Y = 28\cdot 484 + \cdot 01831 X$ years.

We conclude that a small association does exist but that the regression is probably not strictly linear. Turning now to the age of the father, we have the following coefficients :

Age of father at birth of husband, and age of father at birth of wife :

$$r = \cdot 0524 \pm \cdot 0221.$$

Coefficient of contingency $C_2 = \cdot 1954 \pm \cdot 02799$.

If corrected $C_2 = \cdot 0931$ and the correlation ratio for arrays of husbands' fathers

$$\eta = \cdot 1233.$$

If corrected $\eta = \cdot 0997 \pm \cdot 02184$.

The association between age of father at birth of husband and age of father at birth of wife is more marked than in the case of mothers.

TABLE XV.

Age of father at birth of husband.

Age of father at birth of wife							Totals
	20 & under	21—25	26—30	31—35	36—40	41 & over	
20 & under	2	8	9	10	5	3	37
21—25	9	46	39	28	36	25	183
25—30	11	50	69	47	30	22	229
31—35	8	52	81	47	22	19	229
36—40	5	26	28	30	32	15	136*
41 & over	6	24	34	33	27	15	139
Totals ..	41	206	260	195	152	99	953

 $C_{2(r)} = .1954 \pm .02799$. Order of probability about .1. $\sigma_{\text{wife}} = 1.1411$. $\sigma_{\text{husb.}} = 1.3717$.

 $r = .0524$. $\eta_{\text{wife}} = .1223$. $\frac{\sqrt{n}}{.67449} \frac{1}{2} \sqrt{\eta^2 - r^2} = 2.5287$.

* Calculated by Blakeman's Formula.

TABLE XVI.

Age of mother at birth of husband.

Age of mother at birth of wife							Totals
	20 & under	21—25	26—30	31—35	36—40	41 & over	
20 & under	23	32	28	23	20	8	134
21—25	41	101	95	62	35	23	357
26—30	49	91	97	51	39	16	343
31—35	26	90	80	58	25	13	292
36—40	22	31	46	39	19	6	163
41 & over	9	23	30	13	15	5	95
Totals ..	170	368	376	246	153	71	1384

 $C_r = .1654 \pm .0214$. $\sigma_{\text{husb.}} = 1.3554$. $\sigma_{\text{wife}} = 1.3801$. $r = .01798 \pm .0194$.

 $\eta_{\text{husb.}} = .06496$. Linearity = 1.7. Equation $Y = 28.484 + .01831 X$.

If then it can be assumed that these coefficients are not dependent on any statistical fallacy, they lend support to the original proposition, namely that the age at which mating occurs is to some extent determined by the ages of the parents when the parties concerned were born.

I am aware of, and have done my best to emphasise, the defects inherent in the data used in the present enquiry, and a necessary consequence of these imperfections is that several inferences can only be drawn with hesitation.

I think, however, that the concordance of the various results is sufficient to allow me to conclude that

(1) The ages of parents at the time of birth of their offspring are sensibly correlated with the latter's length of life, the sign of the

association being negative. The absolute value of the correlation is, however, small.

(2) This unfavourable influence acts principally at the beginning of life, becomes less marked during the adolescent period and perhaps again becomes prominent at the end of life.

I hope in a subsequent communication to deal with other aspects of this problem.

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ON THE PRESENCE OF SO-CALLED "COMPLEMENT" IN MILK.

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ATTENTION was first directed to the presence of a substance in milk acting as a complement by Pfaundler and Moro⁽¹⁾ and from that time onwards there has been much conflicting evidence published on this subject. Kopf⁽²⁾ stated that complement could not be demonstrated in milk, using the method of Pfaundler and Moro, and the work of Sassenhagen and Bauer⁽³⁾ would seem to show that complement is not present in milk unless the milk of cows suffering from mastitis be present. Sassenhagen⁽⁴⁾ in a later paper states that complement is present in the colostrum of cows and goats and that it disappears as lactation progresses. Lane-Claypon⁽⁵⁾ has followed Pfaundler and Moro and states that complement can be found in almost any milk.

The mere presence or absence of complement in milk is of somewhat academic interest, except from the point of view of the pediatrician, but the stress laid on the fact that its presence is almost diagnostic of mastitis, necessitates a clear understanding of this question.

We have carefully re-investigated the matter using milk from single healthy cows, mixed milk of healthy cows, colostrum, and milk from cows suffering from very slight inflammatory or catarrhal affections of the udder, or possessing abnormal characteristics in one or more quarters. On account of the ease of procedure, we have used the haemolytic

system proposed by Pfaundler and Moro, but employing (at first) the quantities given by Bauer and Sassenhagen, viz. :

1.0 c.c. milk.

0.5 c.c. 5 % suspension of guinea-pig corpuscles.

0.2 c.c. inactivated ox-serum.

From our experiments it is quite clear that much of the conflicting evidence on this subject has been due to the very variable content of amboceptor present in different samples of inactivated ox-serum.

Some preliminary experiments showed that, in general, 0.2 c.c. of the heated serum might be sufficient, but in many cases it was necessary to employ 0.4 or 0.5 c.c. to produce haemolysis in a control using 0.1 c.c. guinea-pig complement.

In any case, it is essential that the ox-blood should be defibrinated and the serum obtained by centrifugalisation as rapidly as possible. After inactivation at 55°, the serum keeps fairly well in the ice chest.

Some preliminary experiments showed that the order of addition of the various members of the haemolytic system was immaterial as was also (within limits) the quantity of guinea-pig corpuscles. The haemolytic effect is entirely dependent on the quantity of amboceptor added. This is seen in the following table :

5% suspension of g. p. corps.	Saline	Heated Ox- serum	Milk	Result*
0.1 c.c.	0.7 c.c.	0.1 c.c.	1.0 c.c.	++++
0.2 „	0.6 „			++++
0.3 „	0.5 „			++++
0.4 „	0.4 „			++++
0.5 „	0.3 „			++++
0.1 „	0.4 „	0.4 c.c.		++++
0.2 „	0.3 „			++++
0.3 „	0.2 „			+++
0.4 „	0.1 „			+++
0.5 „	0.0 „			+++

* The following signs are used to designate the haemolytic effect :

++++ = complete haemolysis

+++ = slight deposit of unlaked cells

++ } = increasing amount of unlaked cells, but pink colour distinct

+ }
— = negative result, no haemolysis.

The milk used in the above case always gave a strong haemolysis with guinea-pig corpuscles and heated ox-serum. It was used, after passing through a separator, as the peculiar action of the fat (as noted

also by Lane-Claypon (*loc. cit.*) is obviated. That the complementary substance in milk is not attached to the fat globules is seen by the following result obtained with the same milk as used above :

Milk or Cream	Saline	Result		
		Whole Milk	Skin Milk	Cream
1.0 c.c.	0.0 c.c.	++++	++++	v. sl. +
0.5 "	0.5 "	+++	+++	—
0.25 "	0.75 "	++	++	—
0.15 "	0.85 "	—	—	—

In all the following experiments recorded the quantities of milk used alone are given. It is to be understood that the quantity was always made up to 1.0 c.c. with saline, that 0.5 c.c. of the 5 % suspension of guinea-pig corpuscles was always employed, and the quantity of heated serum varied from 0.2 c.c.—0.5 c.c. as was required. The tubes used were 75×6 mm. and the order of filling was : (1) milk, (2) saline, if necessary, (3) heated ox-serum, and, after shaking, (4) guinea-pig corpuscles. The contents of the tube were then mixed by inversion two or three times. The tubes were kept in water at 37° C. for 2 hours, the contents being mixed after 30 minutes and 60 minutes, and were finally centrifuged for 10 minutes at 2000 revolutions.

In all cases controls were carried out and as no experiments are recorded in which there was any failure in these, they are not further alluded to.

Cellular elements as a source of complement.

In the greater number of the milks examined, a count was made of the number of cellular elements present, in order to ascertain if there were any connection between these and the presence of complement. The method employed was that described by ourselves and Villar⁽⁶⁾.

It will be seen that there is no general connection to be traced, though, as a rule, any cause which leads to a considerable cell count usually leads to the presence of a "complementary substance." That this latter, however, is not directly due to the increased number of cells is seen from the following experiments :

A large quantity of these cellular elements was obtained from the milk of a cow, whose milk always gave a reaction for "complement" (see cow 24 *infra*), by centrifugalisation, and the cells, after washing twice with saline, were diluted 1 : 20 with saline. The emulsion was

kept for 24 hours at about 5° C. and then tested, but the presence of "complementary substance" could not be detected in 1 c.c. of the emulsion.

COLOSTRUM.

Specimen I.

Heifer, 1st calving. (a) Sample taken 24 hours after calving.

Appearance. As ordinary milk.
Blood. Present in fair quantity.
Cells per c.c. 432,000, many typical large cells present.
Haemolysis. 1.0 c.c. +++, 0.5 c.c. ++, 0.25 c.c. ?, v. sl. +.

(b) Sample taken 72 hours after calving.

Appearance. Normal milk.
Blood. Trace.
Cells per c.c. 224,000.
Haemolysis. Negative.

Specimen II.

Shorthorn cow, 6 years old. (a) Sample taken 12 hours after calving.

Appearance. Very yellow (due to colour of fat), long-chain streptococci present.
Blood. Trace.
Cells per c.c. 6,912,000.
Haemolysis. 1.0 c.c., 0.5 c.c., and 0.25 c.c. all +++, 0.15 c.c. ++.

Mixtures of this colostrum with ordinary milk were made and the presence of 5 % could be easily detected by haemolysis in 1.0 c.c. of the dilution.

This sample of colostrum was kept 24 hours at about 15° C. and again tested with practically similar results.

(b) Sample taken 60 hours after calving.

Appearance. As the 12 hour sample, but not quite so yellow.
Blood. Trace.
Cells per c.c. 11,840,000.
Haemolysis. As in 12 hour sample.

Mixtures of this sample with ordinary milk could be detected down to 10 %.

(c) Sample taken 5 days after calving.

Appearance. As last.
Blood. Not present.
Cells per c.c. 7,072,000.
Haemolysis. 1.0 c.c. +++++, 0.5 c.c. ++, 0.25 c.c. and 0.15 c.c. negative.

Mixtures with milk which had been heated to 55° for 30 minutes gave slight haemolysis in 10 % dilution.

Specimen III.

Heifer, 3 years old. Cross bred Jersey and Shorthorn.

(a) Sample taken 24 hours after calving.

Appearance. As specimen (II), 12 hour sample.
Blood. Slight trace.
Cells per c.c. 720,000.
Haemolysis. 1.0 c.c., 0.5 c.c., 0.25 c.c., 0.15 c.c. all +++++.

Mixtures with an ordinary milk allowed of the detection of 2 % of the colostrum.

(b) Sample taken 3rd day after calving.

Appearance. Somewhat yellow, but otherwise normal milk.
Blood. Not present.
Cells per c.c. 176,000, but many very small cells not included.
Haemolysis. 1.0 c.c. and 0.5 c.c. +++, 0.25 c.c. ++, 0.15 c.c. +.

Mixtures with milk heated at 55° for 30 minutes all gave negative results, there being no haemolysis in any case.

(c) Sample taken 5th day after calving.

Appearance. As last.
Blood. Not present.
Cells per c.c. 160,000, not including many very small cells.
Haemolysis. As last sample.

Mixtures with heated (55°) milk gave a very slight haemolysis in 10 % dilution.

Specimen IV.

From a cow whose previous history was unknown. Quite healthy. The first sample was taken 24 hours after calving. Six hours later the

cow was suffering from an acute attack of milk fever. By suitable treatment recovery rapidly ensued, and 24 hours later she seemed quite normal again.

(a) *Sample taken 24 hours after calving.*

Appearance. Yellow, but otherwise normal milk.

Blood. Not present.

Cells per c.c. Not counted.

Haemolysis. 1.0 c.c. +++++, 0.5 c.c. +++, 0.25 c.c. +, 0.15 c.c. v. sl. +.

Haemolysis, with g.-p. corpuscles only. All tests negative.

*Haemolysis, with sheeps' corpuscles and special amboceptor*¹. All tests negative.

Mixtures of the above with ordinary milk showed a + result in 10 % admixture and a slight haemolysis in 5 % and 2 % admixtures.

(b) *Sample taken 4 days after calving and about 48 hours after recovery from milk fever.*

Appearance, blood, etc. as in sample (a).

Haemolysis. 1.0 c.c. +++++, 0.5 c.c. +++, 0.25 c.c. ++, 0.15 c.c. sl. +.

Haemolysis, with g.-p. corpuscles only. 1.0 c.c. ++. Rest negative.

Haemolysis, with sheeps' corpuscles and special amboceptor. Result doubtful; there appeared to be a very slight haemolysis, but the control, without complement, also showed a distinct though slight haemolysis. It is probable that no real action took place.

Mixtures with ordinary milk showed a + result in 10 % admixture, sl. + in 5 %, and negative in 2 % admixture.

(c) *Sample taken 8 days after calving.*

Appearance. Almost normal milk, only slightly yellowish.

Blood. Not present.

Haemolysis. 1.0 c.c. sl. +. Rest negative.

Haemolysis, with g.-p. corpuscles only. 1.0 c.c. sl. +, rest negative.

Haemolysis, with sheeps' corpuscles and special serum. All tests negative.

¹ This was a very active haemolytic serum obtained by injecting a horse with sheeps' red-blood corpuscles, for a supply of which we are indebted to Messrs Burroughs, Wellcome & Co., per Dr Dale (see also below, p. 495).

Mixtures with ordinary milk were all negative. The cells in this sample were not counted, but there was only a small number present. An analysis of this sample gave the following results: fat 4.10 %, T. solids 13.74 %, N.F.S. 9.64 %, lactose 4.75 %.

From the above results, it is quite evident that colostrum contains a "complementary substance" in considerable quantity and that this persists over a period within which it is quite possible that the milk would be brought into general supply. It is quite useless to lay down a definite period within which such admixture should not be made as the persistence of abnormal milk after parturition varies very considerably in duration, and it is certain that a farmer will use such milk as soon as it is normal in appearance, without any regard to regulations to the contrary. From the above results it is also evident that such admixture may be detectable in other milk, at any rate when present in a dilution of 1 : 10, which might quite easily occur in practice.

MILKS FROM SINGLE COWS IN HEALTHY CONDITION.

The following experiments were made with milk from single cows in perfectly healthy condition, the examinations being made at intervals in some cases. All these cows were in about the 7th month of lactation.

Cow 3. Shorthorn, 5 years old. 10 quarts per day.

- (1) 20th Oct. 1913. Cells per c.c. 160,000.

Haemolysis. All tests negative.

- (2) 25th Nov. 1913. Cells per c.c. 864,000.

Haemolysis. 1.0 c.c. +, others negative.

- (3) 20th Jan. 1914. Cells per c.c. 480,000.

Haemolysis. 1.0 c.c. +++, others negative.

This sample had an extraordinary high acidity when milked, viz. 25.7 degrees Thörner, but was quite normal in taste and appearance.

- (4) 3rd March 1914. Cow nearly dry.

Haemolysis. 1.0 c.c. ++. Rest negative. Using as a control a very sensitive system of sheeps' blood corpuscles and horse serum amboceptor, no haemolysis resulted in 1.0 c.c.

The acidity of the milk was still rather high, 21.3 degrees Thörner. The analysis of this last sample gave, fat 4.65 %, T. solids 14.28 %, N.F.S. 9.63 %, lactose 4.72 %.

Cow 5. Shorthorn, 6 years. 13 quarts per day.

- (1) 20th Oct. 1913. Cells per c.c. 368,000.
Haemolysis. All tests negative.
- (2) 25th Nov. 1913. Cells per c.c. 192,000.
Haemolysis. All tests negative.
- (3) 2nd Dec. 1913. Cells per c.c. 208,000.
Haemolysis. 1·0 c.c. ++, 0·5 c.c. v. sl. +. Rest negative.
- (4) 20th Jan. 1914. Cells per c.c. 128,000.
Haemolysis. 1·0 c.c. v. sl. +. Rest negative.

The acidity in this case also was singularly high, viz. 23·6 degrees Thörner.

- (5) 3rd March 1914. Cow nearly dry.
Haemolysis. All tests negative.
Analysis. Fat 3·50 %, T. solids 12·82 %, N.F.S. 9·32 %, lactose 4·75 %, acidity 21·3 degrees Thörner.

Cow 9. Shorthorn, 9 years.

- (1) 20th Oct. 1913. Cells per c.c. 160,000.
Haemolysis. All tests negative.
- (2) 28th Nov. 1913. Cells per c.c. 80,000.
Haemolysis. 1·0 c.c. + + + +, 0·5 c.c. + + +, 0·25 c.c. + +, 0·15 c.c. + +.

The deposit showed many “ capsular ” bodies and many large cells.

- (3) 3rd March 1914. Cow nearly dry.
Haemolysis. All tests negative.
Analysis. Fat 3·45 %, T. solids 12·96 %, N.F.S. 9·51 %, lactose 4·58 %.

Cow 12. Shorthorn, 7 years.

- (1) 20th Oct. 1913. Cells per c.c. 672,000.
Haemolysis. All tests negative.
- (2) 28th Nov. 1913. Cells per c.c. 304,000.
Haemolysis. 1·0 c.c. + + + +, 0·5 c.c. + + +, 0·25 c.c. + + +, 0·15 c.c. + +.

Cow 15. Shorthorn, 6 years.

- (1) 20th Oct. 1913. Cells per c.c. 608,000.
Haemolysis. All tests negative.
- (2) 28th Nov. 1913. Cells per c.c. 368,000.
Haemolysis. 1·0 c.c. and 0·5 c.c. + + + +, 0·25 and 0·15 c.c. + + +.

- (3) 3rd March 1914. Cow nearly dry.

Haemolysis. 1.0 c.c. ++, rest negative. Control with sheeps' corpuscles and horse serum amboceptor. All tests negative.

Analysis. Fat 3.30 %, T. solids 12.02 %, N.F.S. 8.72 %, lactose 4.26 %.

- Cow 10. Shorthorn, 7 years. Nearly dry.

7th November 1913. Cells per c.c. 656,000.

Haemolysis. 1.0 c.c., 0.5 c.c. and 0.25 c.c. all +.

- Cow 2. Shorthorn, 6 years. Nearly dry.

- (1) 18th November 1913. Cells per c.c. 960,000.

Haemolysis. 1.0 c.c. +++. Rest negative.

- (2) 2nd Dec. 1913. Cells per c.c. 1,440,000.

Haemolysis. 1.0 c.c. ++, 0.5 c.c. v. sl. +. Rest negative.

- Cow 11. Shorthorn. Nearly dry.

18th Nov. 1913. Cells per c.c. 2,960,000.

Haemolysis. 1.0 c.c. +++, 0.5 c.c. +. Rest negative.

- Cow 8. Shorthorn. Nearly dry.

5th Dec. 1913. Cells per c.c. 176,000.

Haemolysis. 1.0 c.c. ++, 0.5 c.c. +. Rest negative.

In the centrifuged deposit of this sample "capsular" bodies were present and many large cells.

Analysis. Fat 3.95 %, T. solids 12.62 %, N.F.S. 8.67 %, lactose 4.08 %, acidity 15.8 degrees Thörner.

- Cow 18. Shorthorn. Nearly dry.

5th Dec. 1913. Cells per c.c. 3,280,000.

Haemolysis. 1.0 c.c. +++, 0.5 c.c. ++, 0.25 c.c. +, 0.15 c.c. —.

Analysis. Fat 3.70 %, T. solids 12.38 %, N.F.S. 8.68 %, lactose 3.61 %, acidity 15.8 degrees Thörner.

- Cow 20. Nearly dry.

19th Dec. 1913. Cells per c.c. 256,000.

Haemolysis. 1.0 c.c. ++, 0.5 c.c. +, 0.25 c.c. v. sl. +, 0.15 c.c. —.

Analysis. Fat 4.45 %, T. solids 13.54 %, N.F.S. 9.09 %, lactose 4.32 %.

Two other cows near the end of lactation were also examined but gave negative results as regards haemolysis. The cell content and analyses of these were:

- (1) Cells per c.c. 2,480,000.
Fat 3.50 %, T. solids 12.46 %, N.F.S. 8.96 %, lactose 3.96 %.
- (2) Cells per c.c. 160,000, many being very large and of indefinite character.
Fat 4.85 %, T. solids 14.34 %, N.F.S. 9.49 %, lactose 4.47 %.

MIXED MILK FROM SEVERAL COWS (8-10).

The samples of these were in all cases examined within 12-20 hours of milking.

(a) *Milks taken during principal calving period.*

- (A) 24th Oct. 1913. Cells per c.c. 432,000.
Haemolysis. All tests negative.
- (B) 6th Nov. 1913. Cells per c.c. 240,000.
Haemolysis. 1.0 c.c. slightly +. Rest negative.
- (C) 8th Nov. 1913. Cells per c.c. 544,000.
Haemolysis. 1.0 c.c. ++. Rest negative.
- (D) 11th Nov. 1913. Cells per c.c. 432,000 containing many large “colostral” bodies.
Haemolysis. 1.0 c.c. ++, 0.5 c.c. +. Rest negative.
- (E) 12th Nov. 1913. Cells per c.c. 464,000.
Haemolysis. 1.0 c.c. +++, 0.5 c.c. ++, 0.25 c.c. v. sl. +, 0.15 c.c. —.
- (F) Same source as (B).
14th Nov. 1913. Cells per c.c. 336,000 containing many “colostral” bodies.
Haemolysis. 1.0 c.c. ++. Rest negative.
- (G) 14th Nov. 1913. Cells per c.c. 304,000.
Haemolysis. 1.0 c.c. ++, 0.5 c.c. sl. +. Rest negative.
- (H) 9th Dec. 1913. Cells per c.c. 1,280,000.
Haemolysis. 1.0 c.c. v. sl. +. Rest negative.
- (I) 20th Jan. 1914. Cells not counted.
Haemolysis. 1.0 c.c. +++++, 0.5 c.c. ++. Rest negative.

Three more samples done during this period all gave negative results. The cell content per c.c. was respectively: (1) 240,000, (2) 768,000,

(3) 770,000. There was no reason to suspect mastitis in any of the above cases; in fact, in the case of (I), in which the strongest haemolytic effect was found, the herd was of a particularly good and healthy character.

Eleven samples of mixed milk were also examined during March 1914. Nine of these gave negative results, and two a very slight positive reaction with 1.0 c.c. of the milk.

MILK FROM COWS HAVING SOME ABNORMALITY OF THE UDDER OR SUFFERING FROM SLIGHT FORMS OF MASTITIS.

The samples of milk examined under this heading were all of such character that the milk would have been used in ordinary dairy work. There was not, in any case, such outward alteration of the milk as would have been noticed during milking. In one or two cases it was noticed that the first stream or two drawn was somewhat thick.

The veterinary notes are appended in brief to each case.

Sample A. Mixed milk of all four quarters of a young half-bred cow apparently quite healthy. Three quarters were normal, but the fourth had an elongated indurated nodule, probably of long standing. Tubercle not found.

Haemolysis. 1.0 c.c. ++, 0.5 c.c. +, 0.25 c.c. v. sl. +, 0.15 c.c. negative.

Sample B. Mixed milk of an old Shorthorn cow, apparently healthy. Three quarters normal, but a slight induration without hypertrophy in the fourth quarter.

Haemolysis. 1.0 c.c. ++, 0.5 c.c. +. Rest negative.

Sample C. Shorthorn cow, having had a fourth calf about two months previously. Milk fell suddenly from 15 quarts to 9 quarts (day previous to sampling). Both hind quarters were slightly swollen. A case of slight catarrhal inflammation, such as would be called a "chill."

The sample was of the whole mixed milk.

Cells per c.c. 30,000,000.

Haemolysis. 1.0 c.c. and 0.5 c.c. +++++, 0.25 c.c. ++, 0.15 c.c. -.

A test was made with the addition of guinea-pig corpuscles alone.

Haemolysis. 1.0 c.c. ++. Rest negative.

Sample D. Shorthorn cow having had fifth calf about two months previously. The left hind quarter was indurated at the upper part, but there was no loss of functional activity. About 16 quarts of milk per day were being given and the cow was in good health. During previous lactation the L.H. quarter was slightly bigger than the R.H. quarter, and there was then probably slight interstitial mastitis, which had become chronic. Sample taken from the L.H. quarter :

Cells per c.c. 608,000.

Haemolysis. 1.0 c.c. + + + +, 0.5 c.c. + +. Rest negative.

The test, with g.-p. corpuscles only, gave negative results.

Sample E. Mixed milk of a cow with slight induration of one quarter probably due to a previous congestion arising from a chill ; it was not due to any recent inflammatory condition. Milk quite normal.

Haemolysis. All tests negative.

Sample F. A "three quarter" cow. One quarter completely atrophied by mastitis during present lactation (probably about three months previous to sampling). Milk normal.

Haemolysis. All tests negative.

Sample G. From a cow with three quarters quite normal. The L.H. quarter had a slight chronic diffused induration probably due to congestion arising from a chill. Not due to any recent inflammatory condition.

(a) *Milk from three normal quarters.*

Haemolysis. 1.0 c.c. + + +, 0.5 c.c. +. Rest negative.

Haemolysis, with only g.-p. corpuscles added. All tests negative.

Haemolysis, with sheeps' corpuscles and special amboceptor. All tests negative.

(b) *Milk from L.H. quarter.*

Haemolysis. 1.0 c.c. + +. Rest negative. With g.-p. corpuscles only and also with special haemolytic system, all tests were negative.

Sample H. In this case the two right quarters of the cow were quite normal, and the milk was also normal. In the two left quarters there was slight mastitis (probably interstitial) of three days' standing. The milk of these quarters was somewhat watery and the fat slightly coagulated.

(a) *Milk of two sound quarters.**Haemolysis.* 1.0 c.c. sl. +. Rest negative.

Tests with g.-p. corpuscles only and also with special system were all negative.

(b) *Milk of two affected quarters.**Haemolysis.* 1.0 c.c. and 0.5 c.c. + + + +, 0.25 c.c. + + +, 0.15 c.c. —.*Haemolysis, with g.-p. corpuscles only.* 1.0 c.c. + + + + (other tests not done).*Haemolysis, using special system.* All tests negative.

It was particularly noticed in this case that the control of the special system using 1 : 10 guinea-pig complement, gave complete haemolysis in ten minutes. Milk has no inhibitory effect on this system, as a parallel control, using inactivated milk to dilute the guinea-pig complement, gave a rapid and complete haemolysis.

Sample I. This was a particularly interesting case, as it was possible to follow it through up to the end of lactation. The cow in question received a kick in the right fore quarter which caused a bruise and some swelling. The milk was outwardly unchanged.

28th Oct. 1913. *Cells per c.c.* 7,040,000.*Haemolysis.* 1.0 c.c. + + +, 0.5 c.c. + +, 0.25 c.c. sl. + 0.15 c.c. —.

4th Nov. 1913. The milk of the separate quarters was examined :

R.H. Cells per c.c. 1,360,000.*Haemolysis.* 1.0 c.c. + +, 0.5 c.c. +, 0.25 c.c. v. sl. +.*Analysis.* Fat 2.25 %, T. solids 11.30 %, N.F.S. 9.05 %, lactose 4.30 %.*R.F. (the bruised quarter). Cells per c.c.* 9,680,000.*Haemolysis.* 1.0 c.c. + + + +, 0.5 c.c. and 0.25 c.c. + + +.*Analysis.* Fat 2.10 %, T. solids 10.08 %, N.F.S. 7.98 %, lactose 3.57 %.*L.H. Cells per c.c.* 4,912,000.*Haemolysis.* 1.0 c.c. + + + +, 0.5 c.c. and 0.25 c.c. + + +.*Analysis.* Fat 2.05 %, T. solids 10.12 %, N.F.S. 8.07 %, lactose 3.77 %.*L.F. Cells per c.c.* 4,992,000.*Haemolysis.* As L.H. quarter.*Analysis.* Fat 2.45 %, T. solids 10.40 %, N.F.S. 7.95 %, lactose 3.67 %.

Tests were made on the following dates :

Date	Haemolysis				Cells per c.c.
	1.0 c.c.	0.5 c.c.	0.25 c.c.	0.15 c.c.	
7th Nov. 1913	++++	++++	++++	+++	7,912,000
11th Nov. ..	++++	++++	++++	+++	5,920,000
18th Nov. ..	++++	+++	++	—	2,992,000
25th Nov. ..	+++	++	+	v. sl. +	4,960,000
2nd Dec. ..	++++	++	sl. +	—	5,760,000
5th Dec. ..	+++	++	sl. +	—	1,344,000
16th Dec. ..	++	+	—	—	4,000,000
16th Jan. 1914	++++	+++	sl. +	—	not done
3rd Feb. ..	—	—	—	—	not done
3rd Mar. .. (practically dry)	++++	++++	+++	—	uncountable

The milk was also tested on Dec. 16th 1913 and March 3rd 1914 with g.-p. corpuscles only, with a negative result in each case.

It was also tested on one occasion with the special haemolytic system with a negative result, and a negative result was obtained with the last sample of March 3rd 1914.

The corresponding analyses of the milk were :

Date	Fat	T. Solids	N.F. Solids	Lactose
28 Oct. 1913	3.17	11.66	8.49	4.05
7 Nov. ..	3.10	9.43	6.33	3.23
12 Nov. ..	3.10	11.52	8.42	3.96
18 Nov. ..	3.30	11.89	8.59	4.26
25 Nov. ..	3.10	11.58	8.48	4.15
5 Jan. 1914	3.50	12.00	8.50	4.28
16 Jan. ..	3.35	11.96	8.61	4.36
3 Mar. ..	3.30	11.99	8.69	1.64

The milk was quite normal in appearance throughout the whole period, except on March 3rd 1914, when the cow was within a few weeks of calving. The milk was then brownish, and had an excess of protein as seen by the analysis. The injury to the udder healed quite readily, and all swelling had disappeared before the end of December. The change in the quality of the milk during and after the injury is easily seen from the above table, the sugar being practically the only constituent affected¹.

From the results recorded above, it is evident that a substance acting as “complement” in a haemolytic system, of which the other members

¹ A sample taken from this cow after her calving, and after colostrum had quite ceased, still showed a powerful haemolytic activity.

are inactivated ox-serum and guinea-pig corpuscles, is present in (a) *colostrum*, both immediately, and for at least six days after parturition, and that this can be detected in 10 % admixture with other milk; (b) *in ordinary milk*, both of single cows, and of several cows, in healthy condition, and that its presence is often very marked in the case of cows which are nearing the end of their lactation period; (c) *in the milk of cows which are suffering from the slighter forms of mastitis or "chill"* not of sufficient severity to cause the milk to be rejected, and also in the milk of cows which have suffered in this manner, and have quite recovered.

Further that the presence of this "complement" is not limited to the quarters which are actually affected but that a "sympathetic" influence is exerted on the sound quarters.

There seems to be no relation between the presence of "complement" and the chemical composition of the milk.

There also appears to be no direct relation between the content of "cellular elements" and the presence of "complement," and in fact the examination made of these, as already stated, would negative any such relation. There appears, however, to be so far a correlation that whatever cause may bring about a rise in the number of these cells will also probably cause the exhibition of, or increase in, the "complementary substance." The general results obtained from the "cell" counts made during this investigation are entirely in accordance with the results obtained previously by ourselves and Villar⁽⁶⁾.

Both persistence of high cell count and of "complementary substance" after injury or slight mastitis are remarkable and not easily accounted for. It must be noticed that it was only in some of the cases of mastitis that two members of the haemolytic system were apparently present, and that haemolysis resulted on the addition of guinea-pig corpuscles alone.

The most remarkable feature of these experiments, however, is that, on no occasion could haemolysis be brought about by the use of a specific haemolytic system of great delicacy. This system consisted of sheep's corpuscles (0.5 c.c. of a 5 % suspension) + horse serum amboceptor (0.1 c.c. of 1 : 30 dilution) of which the minimum haemolytic dose was 0.00066 c.c. With 1 c.c. of a 1 : 10 dilution of guinea-pig complement, haemolysis was complete in five minutes.

This immediately opens up the question as to whether the substance which acts as complement in the natural system (inactivated ox-serum and guinea-pig corpuscles) is *true* complement, as theoretically the

natural system could scarcely be looked upon as being as delicate as the former (specific) system, yet the natural system is capable of producing complete haemolysis when the specific system fails. The questions that this has opened up are being investigated and will form the subject of a further communication. The results obtained so far are somewhat remarkable and point to the fact that, in the case of milk, the natural system (inactivated ox-serum and guinea-pig corpuscles) is specific in character and of great delicacy.

CONCLUSIONS.

When using the haemolytic system consisting of inactivated ox-serum and guinea-pig corpuscles in the quantities stated above, the following conclusions have been arrived at:

1. Milk, from the colostrar stage to the end of lactation, may contain a "complementary substance."
2. Colostral and mastitis milks contain larger quantities of this substance than ordinary milk.
3. In the case of mastitis milk, amboceptor may be present as well as complementary substance, but this would not be detectable in admixture with other milk.
4. Colostrum gives evidence of its presence in 5 % dilution with other milk.
5. Even in the slighter forms of mastitis, "complementary substance" is present in the milk long after subsidence of the inflammatory condition.
6. The presence of "complementary substance" is not specifically diagnostic of mastitis milk.
7. There is no connection to be traced between the number of cellular elements and the presence of "complementary substance," though the appearance of the latter is often accompanied by a rise in the number of the former.
8. The above-mentioned haemolytic system is of extraordinary delicacy in the case of milk.
9. There is some doubt as to the true nature of the "complementary substance" in milk.

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THE FLEAS FOUND ON RATS AND OTHER
RODENTS, LIVING IN ASSOCIATION WITH
MAN, AND TRAPPED IN THE TOWNS, VIL-
LAGES AND NILE BOATS OF UPPER EGYPT.

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Description of Methods of Collection.

THE collections of fleas described in this paper were made during an enquiry into the plague conditions of Upper Egypt, carried out in 1912 and 1913 on behalf of the Egyptian Government. With few exceptions the fleas were taken from rats or other rodents trapped either in the native houses or in the Nile boats (native river craft), which were infested with rats.

The method of trapping was as follows: The trap used was the ordinary wire cage trap with two compartments separated by a partition into which fits a hinged platform with counterweight permitting ingress of the rats, but preventing their escape. The traps containing catches, before being brought from the houses to the laboratory, were enclosed in a black cloth bag to lessen the risk of fleas escaping. At the laboratory the bags were removed and the traps were placed in suitable boxes on the bottom of which were removable metal trays covered with white wax-cloth. Chloroform was poured into the boxes in order to kill the rats and fleas. A considerable number of the fleas were found to have dropped from the rats on to the white cloth, and thus could be easily seen and collected. The chloroformed rats were thoroughly searched for fleas upon a table covered with white cloth.

The most extensive collection in the series is that from Assiut (245 miles south of Cairo), for the reason that in this town trapping operations were carried out continuously during two years. With the idea that the flea population might show seasonal variations as regards species or proportions of species, collections of consecutive periods of two or three months were kept separate, and samples from each lot have been examined, without, however, revealing any seasonal difference.

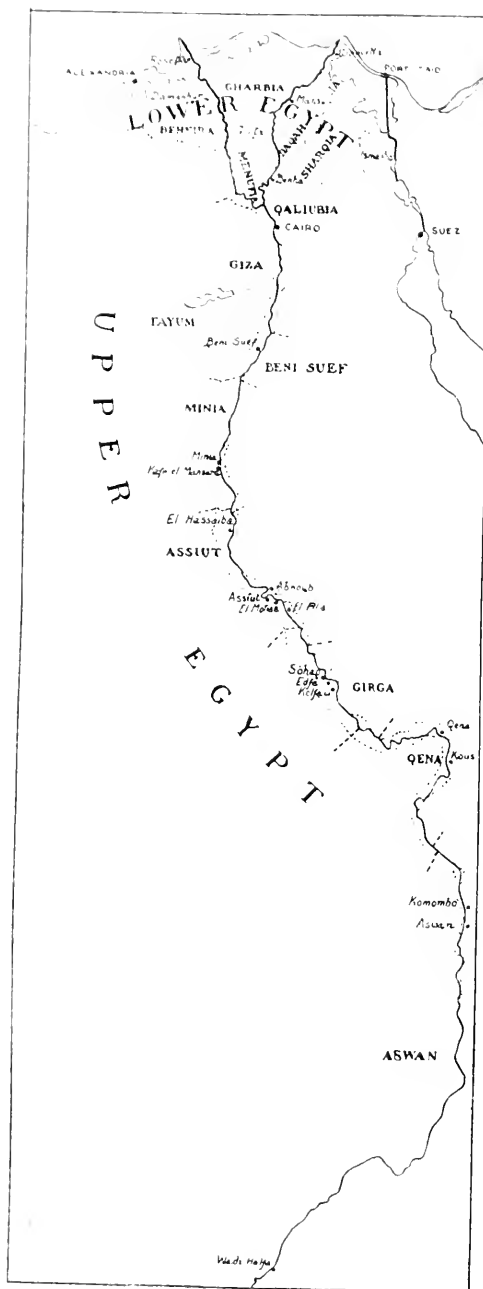
Comments on the Localities from which the Fleas were collected.

The localities represented extend from Cairo to Komombo, an agricultural estate 460 miles south of Cairo, and 26 miles north of Assuan, and are indicated upon the sketch map in the text (p. 500).

With the exception of Cairo and Komombo the towns and villages from which fleas were collected may be regarded as typical for Upper Egypt in respect of the housing conditions. Most of the houses in an Upper Egyptian town or village are constructed of burnt bricks held together chiefly with clay (Nile mud), in which, when dry, rats make their burrows. The earth foundations of the walls are also riddled with rat burrows. As a result rodents infesting houses are in close association with the human inhabitants and with their domestic animals, *e.g.*, dogs, cats and fowls.

The conditions in the quarters in Cairo which yielded the fleas in our collections differ from the above description. The quarters trapped were the Mousky—the native bazaar quarter in the centre of the city, and Boulac, a quarter adjacent to the Nile and opposite to the island of Ghezireh. The houses in these quarters and in Cairo generally are more substantial than those in Upper Egypt, many of them being built of limestone from the neighbouring hills.

From the standpoint of rodent and flea infestation Komombo is of considerable interest. The Komombo estate is a cultivated area of 20,000 feddans (acres), scattered over which are a number (29) of small hamlets each with an average population of about 500 persons. The houses are built, some of stone, others simply of dried mud. The whole estate is of very recent origin, having been developed by means of irrigation from purely desert land only some ten or twelve years ago. *Arvicanthis niloticus* and *Mus norvegicus* are found in the houses and in the fields, but *Mus rattus* and *Acomys cahirinus* have not been met with.

Fleas in Egypt

The Nile sailing boats (feluccas) are built with inner and outer planking bolted to the cross-ribs, thus providing a hollow space in which the rats live. These boats carry cargo—grain, coal, etc.—but also passengers, and ply up and down the Nile from Rosetta and Damietta to Assuan. The rodent infestation on board consists chiefly of *Mus norvegicus*, differing in this respect from the rodent population ashore (Komombo excepted), where this species is, in our experience, a rare inhabitant of houses.

Meteorological data for Cairo, Assiut and Assuan are appended.

TABLE I¹.

Mean monthly meteorological observations recorded at 8 a.m., 2 p.m. and 8 p.m. at Gizeh (Cairo), for the years 1902–10, Assiut 1900–1910 and Assuan 1901–1910.

Mean temperature Centigrade.												
	Jan.	Feb.	March	April	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.
Gizeh near Cairo	10.8	12.3	14.7	19.1	23.1	25.4	26.7	26.5	24.4	21.7	17.0	12.6
Assiut	11.2	13.1	16.6	21.8	26.2	28.8	29.3	29.0	26.2	23.4	17.7	13.7
Assuan	14.5	17.1	20.3	25.4	29.5	32.0	32.6	32.2	30.3	27.4	21.2	16.3
Relative humidity (Saturation 100).												
Gizeh near Cairo	80	72	68	60	56	57	62	67	71	75	78	82
Assiut	69	63	55	43	37	36	40	45	57	62	66	66
Assuan	52	45	40	34	36	34	30	30	35	38	45	49

Determination of the Species.

In the case of small collections every individual flea in the tube was separately examined under a compound microscope, but of the large collections, comprising from 2000 to 10,000 fleas in a single tube, a sample only was examined. These samples consisted of at least 500, and in some instances of 1000 to 2000 specimens, each of which was identified. The number of specimens noted in the Tables as examined must not be taken as affording any guide to the relative numbers captured on the various individual hosts mentioned, nor as evidence of a seasonal or geographical prevalence. The statistics relating to

¹ In tabulating our results we have followed on the lines laid down by Chick and Martin (1911), the present paper forming an addendum to their work and filling the gap caused by the fact that there were no figures relating to Egyptian fleas available at the time they wrote.

this side of the research are in course of arrangement and will be published later.

We take this opportunity of recording our thanks to Dr Harriette Chick for assistance with the earlier consignments, and to the Hon. N. Charles Rothschild for his kindness in checking some of our determinations of species, especially in regard to *Xenopsylla chephrenis* and *X. cleopatrae*.

Notes on the Species represented in the Collections.

Xenopsylla cheopis, as was to be expected, makes up by far the greatest bulk of the collections as a whole. When the animals from which the fleas were taken were trapped on land the percentage of this species normally present varies from 90 % to 100 %.

The few exceptions to this general rule are afforded by such hosts as *Gerbillus pyramidum*, a specimen caught wild, yielding a high percentage of *Xenopsylla cleopatrae*, a species of hedgehog which showed a distinct preponderance of *Ctenocephalus felis*, a weasel carrying *Echidnophaga gallinaceus* and *Acomys cahirinus* from Cairo, which had a marked infestation of *Xenopsylla chephrenis*. On hosts trapped in the Nile boats the relative numbers of *X. cheopis* declined, their place being occupied by *Leptopsylla musculi*.

Leptopsylla musculi. It is remarkable how dependent the occurrence of this species in the collection is upon the trapping of rodents from the Nile boats (feluccas) during the cooler season of the year.

The percentage captured upon animals in dwellings is infinitesimal, whereas in the collections from feluccas it sometimes exceeds that of *X. cheopis*, rising in one instance to 85 %.

It is no easy matter to disentangle and assess correctly the various factors which may contribute to this result. As already pointed out in our remarks on localities, *M. norvegicus* is the dominant rodent on the Nile boats.

Further, from a considerable mass of data relating to the rat and rat flea infestation of Lower Egypt collected by Professor Bitter¹ and Dr Charles Todd of the Hygienic Institute in Cairo, it would appear that *M. norvegicus* occurs much more commonly as a house-rat in Lower Egypt than in Upper Egypt, and also that *L. musculi* is a much more

¹ We desire to acknowledge the courtesy of Professor Bitter and Dr Charles Todd in permitting us the privilege of becoming acquainted with their observations.

TABLE II.
Showing species of Fleas found on various Hosts trapped in Houses.

Locality			Date of Collection	Species of Animal	Number of specimens examined	Percentage of each species of Flea									
Town or Village	District (Markazi)	Province (Mudiriya)				<i>Pulex irritans</i>	<i>Echidnophaga gallinacea</i>	<i>Xenopsylla cheopis</i>	<i>Xenopsylla cheopis</i>	<i>Xenopsylla cheopis</i>	<i>Ceratophyllus fasciatus</i>	<i>Ceratophyllus fasciatus</i>	<i>Leptopsylla muscivora</i>		
Cairo			Nov. 1913	<i>Mus rattus</i>	208	—	—	91.8	8.1	—	—	—	—	—	
			Nov. 1913	<i>Acomys cahirinus</i>	212	—	—	3.3	96.2	—	—	—	—	—	
Kafr-el-Mansura	Minia	Assiut	May-Aug. 1913	Weasel	45	—	—	—	—	—	—	—	—	—	
			Dec. 1911	<i>Mus rattus</i>	114	—	—	99.1	—	—	—	—	—	—	
El Hassarba	Assiut	Assiut	Sept. 1913	<i>Mus rattus</i>	540	—	—	100	—	—	—	—	—	—	
				<i>Mus norvegicus</i>	89	—	—	94.3	—	—	—	—	—	—	
Abnoub	Assiut	Assiut	Jan. 1912	<i>Mus rattus</i>	1058	0.7	—	98.9	—	—	—	—	—	—	
				<i>Mus norvegicus</i>	1	—	—	1 flea only	—	—	—	—	—	—	
Assiut	Assiut	Assiut	Dec. 1911 to Jan. 1913	<i>Mus rattus</i>	13,131	0.35	—	98.95	—	—	—	—	—	—	
				<i>Mus norvegicus</i>	2664	0.37	—	97.94	—	—	—	—	—	—	
El Filia	Assiut	Assiut		<i>Acomys cahirinus</i>	5839	0.47	—	98.25	0.74	—	—	—	—	—	
				<i>Arviculantis niloticus</i>	2060	0.09	—	98.8	—	—	—	—	—	—	
Elfa and Kelfaw	Sohag	Girga	July, Aug. 1913	<i>Crocidura olivieri</i>	5	—	—	(5)	—	—	—	—	—	—	
				<i>Mus rattus</i>	623	—	—	100	—	—	—	—	—	—	
Elfa and Kelfaw	Sohag	Girga	July 1912	<i>Acomys cahirinus</i>	196	—	—	100	—	—	—	—	—	—	
				<i>Mus rattus</i>	185	—	—	100	—	—	—	—	—	—	
Kous and neighbouring villages	Kous	Kena		<i>Acomys cahirinus</i>	125	—	—	100	—	—	—	—	—	—	
				<i>Arviculantis niloticus</i>	32	—	—	96.8	—	—	—	—	—	—	
Kous and neighbouring villages	Kous	Kena	April to June 1912	<i>Crocidura olivieri</i>	11	—	—	100	—	—	—	—	—	—	
				<i>Mus rattus</i>	845	0.1	—	99.8	—	—	—	—	—	—	
Kous and neighbouring villages	Kous	Kena		<i>Mus norvegicus</i>	325	—	—	100	—	—	—	—	—	—	
				<i>Acomys cahirinus</i>	424	0.9	—	99	—	—	—	—	—	—	
Kous and neighbouring villages	Kous	Kena		<i>Arviculantis niloticus</i>	738	0.4	—	99.5	—	—	—	—	—	—	
				<i>Mus norvegicus</i>	932	—	—	99.9	—	—	—	—	—	—	
Komombo	Assuan	Assuan	Nov. 1911 to Feb. 1912	<i>Arviculantis niloticus</i>	1754	—	—	99.9	—	—	—	—	—	0.1	
				<i>Gerbillus pyramidalis</i>	14	—	—	100	—	—	—	—	—	0.05	
Komombo	Assuan	Assuan		<i>Gerbillus pyramidalis</i>	12	8	—	91.6	—	—	—	—	—	—	
				Mouse	12	—	—	91.6	—	—	—	—	—	—	

The number in brackets records the actual specimens and is not a percentage.

TABLE III.

Showing the number and species of Fleas taken from Rodents trapped on Feluccas (Nile boats) at Abnoub, Assiut and Komombo.

Locality		Province (Markaz)	Date of Collection	Species of Animal	Number of specimens examined	Percentage of number of each species of Flea						
Town or village	District					<i>Pulex irritans</i>	<i>Echidnophaga gallinacea</i>	<i>Xenopsylla cheopis</i>	<i>Xenopsylla chephrenis</i>	<i>Xenopsylla cheopaterae</i>	<i>Ceratophyllus fuscicornis</i>	<i>Ctenocephalides felis</i>
Abnoub	Assiut	Assiut	Jan. 1912	<i>Mus norvegicus</i> and <i>rattus</i>	32	3.1	—	56.2	—	—	—	6.2
				<i>Acomys cahirinus</i>	5	(1)	—	(1)	—	—	—	34.3
				<i>Mus rattus</i>	585	0.8	—	60.2	—	—	—	0.1
				<i>Mus norvegicus</i>	3261	0.1	0.1	49.3	0.03	0.06	—	0.1
				<i>Acomys cahirinus</i>	79	3.7	—	78.4	—	—	—	17.7
				<i>Mus rattus</i>	143	—	1.4	97.9	—	—	—	0.7
				<i>Mus norvegicus</i>	478	—	—	98.7	—	—	—	1.2

Numbers in brackets record the actual specimens, not percentages.

TABLE IV.

Showing that *Leptopsylla musculi* is relatively more numerous than *Xenopsylla cheopis* during the cooler months of the year.

Fleas taken on Rodents trapped on Feluccas (Nile boats) at Assiut.

Date of Collection		Species of Animal	Number of specimens examined	Percentage of each species of Flea						
				<i>Pulex irritans</i>	<i>Echidnophaga gallinacea</i>	<i>Xenopsylla cheopis</i>	<i>Xenopsylla chephrenis</i>	<i>Ceratophyllus fuscicornis</i>	<i>Ctenocephalides felis</i>	<i>Leptopsylla musculi</i>
Feb., March 1912		<i>Mus rattus</i>	319	1.5	—	48.5	—	—	0.3	49.5
June, July, Aug. 1912			206	—	—	99.5	—	—	—	0.4
Sept., Oct. Nov., Dec. 1912			60	—	—	73.3	—	—	—	26.6
Feb., March 1912		<i>Mus norvegicus</i>	1458	0.1	0.06	13.4	0.06	0.06	0.2	85.9
April, May, June 1912			536	0.1	0.1	74.8	—	0.1	—	24.6
July, Aug., Sept. 1912			262	0.3	0.3	99.2	—	—	—	—
Oct., Nov., Dec. 1912			902	—	0.1	79.7	—	—	0.1	20.0
Jan. 1913			103	—	—	73.3	—	—	—	26.6
Feb., March 1912		<i>Acomys cahirinus</i>	32	(1)	—	(18)	—	—	—	(13)
April, May, June 1912			34	(2)	—	(32)	—	—	—	—
July, August, Sept. 1912			3	—	—	(3)	—	—	—	—
Oct., Nov., Dec. 1912			10	—	—	(9)	—	—	—	(1)

Numbers in brackets record actual specimens, not percentages.

TABLE V.

Showing species of Fleas obtained from a variety of sources not included in the previous Tables.

Locality			Date of Collection	Province (Mudiria)	Host or Source from which Fleas were obtained	Number of specimens examined	Percentage or number of each species of Flea						
Town or Village	District	Province (Mudiria)					<i>Palor-teridans</i>	<i>Echidnoplaga galinavens</i>	<i>Xenopsylla cheopis</i>	<i>Xenopsylla cheopurens</i>	<i>Xenopsylla cleopatrae</i>	<i>Ceratophyllus placidus</i>	<i>Ctenocephalides felis</i>
Assiut	Assiut	Assiut	May 1913	Assiut	Dog	284	4.9	—	—	—	—	—	—
Assiut	Assiut	Assiut		Assiut	Human host on clothing								
Abnoub	Abnoub	Assiut	Jan. 1912	Assiut	<i>Mus rattus</i> and mouse	538	100	—	—	—	—	—	—
Abnoub	Abnoub	Assiut	Jan. 1912	Assiut	<i>Mus rattus</i> and mouse	4	—	—	(4)	—	—	—	—
Abnoub	Abnoub	Assiut	Jan. 1912	Assiut	<i>norvegicus</i>	431	0.6	—	93.7	—	—	—	5.5
El Motiaa	Assiut	Assiut		Assiut	Clothes of plague patient	44	(43)	—	(1)	—	—	—	—
Kous	Kous	Kena	May 1912	Kena	Hedgehog	18	(1)	—	—	—	—	—	(17)
Komombo	Assiut	Assiut		Assiut	Clothes of plague patient	1	(1)	—	—	—	—	—	—
Komombo	Assiut	Assiut	Nov. 1911	Assiut	<i>Acomys</i> from Nile bank	14	—	—	—	—	—	—	—
Komombo	Assiut	Assiut	Feb. 1912	Assiut	<i>Gerbillus pyramidum</i>	20	—	—	(14)	—	—	—	—
Komombo	Assiut	Assiut	Feb. 1912	Assiut	Rats' nests in houses	121	—	—	(2)	—	(18)	—	—
Komombo	Assiut	Assiut	Feb., Mar. 1912	Assiut	Plague infected houses	2821	—	—	100	—	—	—	—
Komombo	Assiut	Assiut	Feb. 1912	Assiut	Clothes of rat catchers in infected houses				100	—	—	—	—
Komombo	Assiut	Assiut	Feb. 1912	Assiut	Clothes of plague patients	193	4	—	96	—	—	—	—
Komombo	Assiut	Assiut	Feb. 1912	Assiut	Clothes of plague patients	15	(2)	—	(13)	—	—	—	—

Numbers in brackets record actual specimens, not percentages.

prevalent species of flea on house-rats in Lower Egypt than in Upper Egypt.

From these considerations it might be inferred that the occurrence of *L. musculi* and of *M. norvegicus* are inter-related, but on the other hand this view is not supported by any evidence that *L. musculi* favours *M. norvegicus* rather than *M. rattus*, whether the animals are trapped in feluccas in Upper Egypt or in the native dwellings of Lower Egypt.

The two most plausible solutions are either that *L. musculi* is a species mainly restricted to Lower Egypt and that the feluccas are infested from this source or that the conditions on the Nile boats are more favourable for it than those in the rat-nests on land in Upper Egypt.

It seems definite, however, that the land conditions in Upper Egypt are unfavourable to the continued existence of *L. musculi*, though whether because of a small mouse population, or owing to the climatic conditions or for some other reason, is not clear.

Ceratophyllus is represented in these collections by three specimens of *fasciatus*, all taken from *M. norvegicus* trapped on feluccas during the first half of the year. From these circumstances and the fact that the species is present in small numbers on rats captured in Lower Egypt, we again get a suggestion of importation from the North, but curiously it is *M. rattus* which carries the larger number of specimens according to the unpublished data we have been privileged to see.

Xenopsylla cleopatrae was found only on one occasion, the host being a *Gerbillus pyramidum* captured in February, 1912, at Komombo. This flea would appear to be in no way associated with the usual rodent population of inhabited districts.

Xenopsylla chephrenis. Of the 265 specimens of this flea found in these collections, by far the larger number (221) are from Cairo; the remainder (44 specimens) were captured at Assiut. Of these latter 43 were on *Acomys* trapped in houses and one from a *M. norvegicus*, caught on a Nile boat at that town. The distribution on the hosts of the species trapped at Cairo is also significant; 204 of the fleas being found on *Acomys*, the remaining 17 on *M. rattus*. See Table II.

Ctenocephalus felis. Of the genus *Ctenocephalus*, *Ct. felis* alone is present in these collections, and although the percentage is low in comparison with that of *Xenopsylla cheopis* or *Leptopsylla musculi*, the actual number of specimens is large enough to justify the expectation of finding *Ct. canis* also, if this species occurs in Egypt. Probably,

as in other tropical countries, *Ct. felis* does duty for both feline and canine animals in Upper Egypt.

Echidnophaga gallinacea is rare considering the probable chances of intercourse between rats and fowls.

Pulex irritans. In view of the close association obtaining between rats and man under the conditions in which a large section of the human population of Egypt live, the percentage of this species found on trapped rats must be considered as extremely small, especially as human infestation with *Pulex irritans* is sometimes very heavy (Table V).

SEX PROPORTIONS. The very marked discrepancy between the number of male and female specimens of *Xenopsylla chephrenis* caused us to keep a record of the sexes of other species in the tubes that had not previously been examined when this discrepancy was first noted. The results set forth in the following table show that some difference in the proportion of the sexes is also present in *X. cheopis*, although to a much slighter extent, the difference only amounting to 7 %.

TABLE VI.

Proportion of sexes in fleas captured on Rats and other Rodents, and on Man.

Species	Number examined	Males	Females	Excess
<i>Xenopsylla cheopis</i>	8498	3948 (46.5 %)	4550 (53.5 %)	602 (7 %)
<i>Xenopsylla chephrenis</i>	259	36 (13.9 %)	223 (86.1 %)	187 (72.2 %)
<i>Pulex irritans</i>	547	329 (60.1 %)	218 (39.9 %)	111 (20.2 %)

As the numbers dealt with in arriving at this conclusion are considerable, over 8000 specimens from 18 parcels collected either at different seasons or in separate localities, it is probably justifiable to suppose that this want of balance is general and implies some variation in the habits of the sexes. With regard to *X. chephrenis*, although the number of specimens is not large, the discrepancy is so striking that there seems no doubt that some deep-seated divergence in the habit of the sexes is indicated. The explanation we would suggest is that the males wait in the nest to fertilize the females as they emerge from their cocoons, while the instinct of the female is to attach herself more closely to the host and feed as continuously as possible, as by this procedure egg development will be more rapid, and the chances of founding new colonies when the young of the host scatter will be enhanced.

In the terms of such a theory the position of *X. cheopis* is either that of a species in which the ancestral habit (possibly originating in a desert habitat) is waning with its adaptation to semi-domesticated hosts, or, which seems a less plausible proposition, that *X. chephrenis* is developing to excess the ancestral habits of the group which are retained by *X. cheopis*.

In the case of *P. irritans* it is doubtful as to how far the excess of males is a chance phenomenon, as the numbers, which are not large, relate to two parcels only.

REFERENCE.

- CHICK, H., and MARTIN, C. J. (1911). The fleas common on rats in different parts of the world and the readiness with which they bite man. *Journ. of Hygiene*, XL 122.

THE STERILITY OF NORMAL URINE IN MAN.

BY EDWARD C. HORT, F.R.C.P. Edin.

(From the Lister Institute.)

It appears to be widely believed that normal human urine as voided is sterile, and that the fact of sterility is capable of ready demonstration.

The main grounds for this belief are the well-known experiments on the subject by Lord Lister, and the innumerable negative cultural results that have since been obtained after inoculation of laboratory media with unincubated urine or its centrifugal deposits.

When, however, the experiments of Lord Lister are carefully studied it is at once apparent that they do not prove the sterility of normal urine, as he would have been to-day the first to acknowledge. For example many of the specimens of urine that he examined for evidence of sterility were allowed to stand at room temperature for considerable periods, and if no organisms were then found, or if the urine remained clear, it was assumed that none were present.

Evidence of this nature, however, is on theoretical grounds hardly admissible because incubation of the urine at body temperature might conceivably be necessary to allow of multiplication of infecting organisms to take place. This possible explanation of Lord Lister's deductions from his experiments on the sterility of normal urine was therefore put to the following simple test.

Experiment 1.

An ordinary specimen of male urine from a healthy subject was received into two sterile flasks, which were at once closed with sterile rubber corks. Flask 1 was incubated at body temperature for 48 hours, and its contents were then found to be turbid from the presence of large Gram positive dividing cocci. Flask 2 was allowed to stand at room temperature for 10 days, and at the end of this time the urine was found to be perfectly clear. This flask was then placed in the incubator for 48 hours, and the contained urine was at the end of this time found to be turbid from the presence of large Gram positive dividing cocci. Two peptone agar tubes, which had each been inoculated with the invisible centrifuged deposit of 5·0 c.c. of the clear

urine immediately after its collection, were free from colonies at the end of a period of 14 days, during which incubation at 37·0° C. had been allowed to proceed without interruption.

From this experiment it is clear that the absence of turbidity in unincubated urine after a lapse of several days does not necessarily indicate its sterility, and evidence of this nature therefore cannot be held to be satisfactory.

There remains for consideration the evidence afforded by the negative cultural results that often follow the inoculation of laboratory media with unincubated urine or its centrifuged deposits.

On theoretical grounds it would appear unsafe to assume the sterility of specimens of normal urine merely from failure to infect the ordinary laboratory media. For example, a given specimen might contain organisms which were unable to adapt themselves to a new environment, or could only do so if firmly established on urine first. And this would particularly apply to attempts to inoculate solid media. Moreover, the possibility of an infection of the total volume of urine discharged, for example, in 24 hours could hardly be excluded by failure to cultivate from the small fractional samples usually placed into tubes containing nutrient agar, or capable of reception by the ordinary centrifuge. And finally the degree of infection of normal urine might be so slight as to escape detection in the case of centrifuged deposits from clear urine unless these were transferred without loss to the medium employed as indicator. And this is a difficult feat to achieve.

Notwithstanding these theoretical objections to the use of laboratory media as indicators of the absence of infection of normal urine, the frequent negative results obtained are widely quoted as evidence of its absolute sterility. The value of laboratory media as indicators of sterility was therefore submitted to the following tests.

Experiment 2.

Clear urine from a healthy male was discharged into two sterile tubes, each receiving about 15·0 c.c. Tube 1 plugged with sterile wool was incubated at body temperature for 48 hours, and was then found to contain urine turbid from the presence of dividing cocci in groups. Tube 2 immediately after filling was centrifuged for 15 minutes in a high speed machine, and the supernatant urine carefully pipetted off. As much as possible of the slight deposit of mucus was then transferred in a platinum loop to two agar tubes, which were incubated for 14 days, and examined daily. One tube remained sterile throughout, the second showing one colony of cocci at the end of 76 hours, no further colonies appearing within 14 days.

From this experiment, confirming a similar observation in Experiment 1, it is clear that the inoculation of a solid medium such as nutrient agar with the centrifuged deposit from normal urine cannot be relied on to demonstrate its sterility.

Experiment 3.

The same conditions were observed as in Experiment 2 with the exception that the centrifuged deposit was transferred to two tubes of broth. The control tube of uncentrifuged urine and one of the tubes of broth were found to be turbid with growth after incubation for 48 hours, whilst the second tube of broth was still clear at the end of 21 days.

This experiment suggests that it is not safe to assume the sterility of a sample of normal urine from the absence of growth in broth inoculated with the centrifuged deposit of clear urine.

It may, however, be reasonably objected that the simplest method of determining if a given specimen of urine is sterile or not is to inoculate broth or agar, or other suitable medium, with fractional volumes of uncentrifuged urine. The validity of this objection was therefore tested.

Experiment 4.

Clear urine was discharged into a sterile flask, and 1.0 c.c. was at once pipetted over the surface of the medium in each of three agar tubes, the flask and the three tubes being subsequently incubated at body temperature for 76 hours, care being taken to prevent movement of the urine in the agar tubes during incubation. At the end of 76 hours the surfaces of agar were free from colonies, whilst the urine in the flask and in the three tubes was turbid with dividing cocci.

This experiment shows that if an inoculum of urine in an agar tube be undisturbed during incubation a fallacious result may be obtained if the laboratory medium alone be used as the indicator of sterility of the inoculum. And it also suggests that the urine is a more sensitive indicator of its own infection, as might be expected from its liquid nature, than is nutrient agar.

Experiment 5.

Clear urine from a healthy male was next discharged into a sterile test tube, and two tubes of agar were each inoculated with 1.0 c.c. of the urine obtained, a different pipette being used for each inoculum. During incubation the urine was repeatedly flushed over the surface of the agar, and in 48 hours each surface was covered with countless colonies of large dividing cocci. The control tube containing incubated urine alone also contained at the end of 48 hours large dividing cocci.

This experiment suggests that once the organisms found had firmly established themselves on urine they had no difficulty in adapting themselves to the solid medium if opportunity were allowed for re-inoculation.

Experiment 6.

Clear urine was then discharged direct into tubes of broth, and in 48 hours the incubated mixture was in the three tubes inoculated turbid with large dividing cocci.

Taken as a whole these six experiments suggest that the evidence hitherto relied on in favour of the sterility of normal urine should be abandoned, and that the fact of sterility of normal urine, if it be a fact, still awaits demonstration. And they also suggest that urine when incubated may be a better and more convenient indicator of its infection in the fresh state than the ordinary laboratory media whether liquid or solid.

If, however, incubated urine be used as the indicator of its absolute sterility, complete exclusion of extraneous organisms during the process of collection is essential. Unfortunately this is not an easy matter owing to our ignorance of the commonest source of extraneous infection, the difficulty of collecting human urine in such a way as to exclude it, and the time required in any given case to determine whether organisms found in, or cultivated from, the urine are extraneous or not. By extraneous infection is here meant infection which occurs after the escape of urine from the bladder. The possible sources of infection of male urine (with which alone this paper is concerned) occurring during and after discharge appear to be the urethra including the vestibule, the surface of the glans and of the lips of the meatus, the air through which the urine is collected, and the receiving flask. In all the experiments here cited, unless otherwise stated, infection from the receiving flask was excluded by exposing it to dry heat at 180·0°C., each flask being plugged with wool before being heated. Infection from the urethra and vestibule and from the lips of the meatus was excluded as far as possible by adoption of the following technique. The surfaces of the glans and of the lips of the meatus were washed with sterile wool soaked in a 1 in 1000 solution of perchloride of mercury in water. The glans was then immersed in this solution for one minute, a few ounces of urine being finally discharged under the surface of the solution in order to flush the urethra and vestibule. Assuming these precautions to be efficient, the only two sources of extraneous infection which remain are the urethra, including the vestibule in so far as it cannot be

cleansed by the flushing referred to of organisms that are supposed normally to inhabit it, and the air through which discharge takes place.

In spite of these precautions however, as already shown, it was not found possible to obtain a sterile specimen of urine. The two methods of exclusion of extraneous infection usually relied on by different observers are the serial method of collection and the catheter method. Both these methods were, therefore, carefully tested in order to determine if by the adoption of either of them sterile specimens of normal urine could be obtained, using as the indicator of sterility the incubated urine itself.

By the serial method is here meant collection into a numbered series of flasks, or other receptacle, only the last members of the series being reserved for study.

Experiment 7.

After careful washing of the genital surfaces and after preliminary flushing of the urethra under perchloride of mercury, urine was discharged through air by a healthy male into a series of narrow mouthed flasks numbered 1 to 4. After incubation for 48 hours each flask was found to be turbid with organisms. Each flask contained about 100·0 c.c. of urine.

This experiment was repeated by the same individual five times in one laboratory, once in a second laboratory and twice in a private house. Of the 24 flasks incubated not one remained sterile at the end of three days. All were turbid, the organisms found in each series being constant to the site of collection of each series, but differing in each site. One series, for example, in one laboratory gave small cocci only in groups, a second in a different laboratory gave a short bacillus, cocci in groups and chains, a streptothrix and yeast-like cells, and a third series gave large cocci alone.

Experiment 8.

In this experiment urine was discharged by a healthy male into a series of four numbered filter flasks, the side piece being plugged with sterile wool. In each case the glans was placed within the mouth of the flask to exclude air as far as possible, and each flask was finally closed with a sterile rubber cork. At the end of a period of incubation lasting ten days each flask, containing about 60·0 c.c. of urine, was found to be heavily infected. This experiment was repeated 15 times by ten healthy subjects with varying volumes of urine, extreme care being taken with the preliminary toilet of the genital surfaces and vestibule. Of a total of 64 flasks incubated for 14 days not one contained sterile urine at the end of that time. Great irregularity was noted as to the time of appearance of turbidity, which was often independent of the position of a flask in the series, even when the volumes were constant.

Experiment 9.

Into three series of flasks, each series containing 12 members, were collected, in the same way as in Experiment 8, volumes of urine varying from 10 to 40 c.c. Of the 36 specimens of urine incubated not one was found to be sterile at the end of 14 days.

Thus out of a total of 128 flasks of normal urine in these experiments a sterile specimen was not once obtained.

There are three alternative interpretations of these results:

1. Normal urine, contrary to general belief, is not sterile when it leaves the bladder, that is, before it enters the urethra.

2. The serial method of collection cannot be trusted to exclude urethral organisms from urine during its passage along the urethra, assuming such organisms to exist in the healthy subject.

3. A common source of extraneous infection of urine is the air through which it passes after it has left the urethra.

The experiments, in other words, throw no certain light on the source or time of infection of the various samples of urine examined, though from Experiment 7 the possibility of air infection is certainly suggested. They do, however, clearly show that the serial method of collection through unsterilized air is of no value in attempting to obtain sterile specimens of normal urine.

The catheter method of collection was next examined.

Experiment 10.

Specimens of normal urine from ten healthy males were collected by catheterization, the urine being discharged through air in the ordinary way, the free end of the catheter being finally placed inside sterile flasks. The catheters were sterilized before use in liquid paraffin in the autoclave at a temperature of 125·0° C. in order to avoid the necessity for lubrication after sterilization¹. In each case the first few ounces of urine discharged were rejected before the flasks were filled. After incubation of ten specimens obtained in this way turbidity due to the presence of organisms was present in all at the end of 14 days.

This experiment shows that the catheter method of collection as generally employed is no more reliable than is the serial method in securing sterile specimens of normal urine.

An attempt was therefore made to obtain a sterile catheter specimen by only allowing the urine to pass through sterilized air as it leaves the catheter.

Experiment 11.

A catheter specimen of urine was collected from a healthy male, the free end of the catheter, fitted with a clamp, being attached to the side piece of a filter flask before sterilization, the mouth being closed with a sterile wool plug. As soon as the

¹ Absolute sterilization being impossible to ensure by this method in the case of the presence of spore-bearing organisms, these were carefully searched for in the catheter specimens of urine obtained. Only non spore-bearing organisms were however cultivated.

specimen was obtained, but before withdrawal of the catheter from the bladder, the clamp was closed and the catheter cut off. After incubation for 21 days the urine was found to be clear, and appeared to be sterile so far as could be judged from film preparations of the centrifuged deposit and from inoculation of broth.

Immediately after separation of the catheter from the flask a few ounces of urine were drawn through unsterilized air into a second flask, and in 48 hours the urine was found to be turbid with growth of large dividing cocci.

This experiment, taken alone, is of little value as evidence of the sterility of normal urine in general, but it suggests that a normal specimen can be obtained in an apparently sterile condition if passed through sterile air.

That unsterilized air may be a factor in extraneous infection of urine during its collection is also suggested by an experiment of a different nature.

Experiment 12.

A specimen of urine passed in the ordinary way without any preliminary toilet was collected in a sterile flask. Twenty c.c. of the urine were then transferred in a sterile pipette into a test tube. This was at once boiled, and when cool ten c.c. were poured through air into a second tube. The flask and the two tubes were then placed in the incubator for 48 hours. The flask and the tube containing the poured urine were both infected with large Gram positive dividing cocci, whilst the second tube containing the boiled urine which had not been poured remained clear and sterile at the end of a month. This experiment was repeated three times with the same results.

CONCLUSIONS.

1. As an index of the sterility of normal urine absence of growth on the ordinary laboratory media inoculated with fresh urine or its centrifuged deposit is of little or no value, except in the case of broth inoculated with uncentrifuged urine.
2. The use of incubated urine as the medium of growth shows it to be a highly sensitive and reliable indicator of infection with organisms that appear to be extraneous in the sense defined.
3. It is difficult, if not impossible, to obtain sterile specimens of normal urine by the serial method or the catheter method of collection as ordinarily carried out.
4. Unsterilized air through which urine passes during the process of collection appears to be a frequent source of the extraneous infection of urine, whether the catheter or serial methods be employed or not.
5. The sterility of normal urine in man still awaits demonstration.

SUPPLEMENTARY NOTE BY EDWARD C. HORT
AND W. W. INGRAM.

Working in conjunction we have repeated on a large scale involving several hundred observations the experiments above described, and have without exception obtained the same essential results.

It appears that the most convenient method of determining whether normal human urine is or is not sterile before it leaves the bladder would be to obtain a sample by suprapubic puncture during life. This procedure has obvious disadvantages in man. We have therefore applied a modification of the method to animals by puncturing the bladder immediately after death with the following results:

Owing to the kindness of the authorities of the Metropolitan Cattle Market we were able to examine the bladder urine of fourteen animals. Immediately after the death of the animals the bladder was exposed, its surface being then seared with a hot iron. A sharp sterile glass pipette was then pushed through the wall of the bladder and a few cubic centimetres of urine drawn up. The pipette was in each case at once sealed in the flame and within an hour placed in the incubator and kept there for two and a half months at a temperature of 37.0°C. Companion pipettes were withdrawn from the incubator at intervals and examined. The contents of these pipettes and of those kept in the incubator for two and a half months were perfectly clear and in no case could organisms be discovered in the centrifuged deposits, and in no case did inoculation of laboratory media with volumes of the incubated urine reveal infection.

We then repeated these observations with the urine of twelve apparently healthy guinea pigs, using the same technique. In each case the specimens examined remained sterile as far as could be judged by examination of the centrifuged deposit and by attempts at cultivation on the ordinary laboratory media inoculated with the incubated urine.

We conclude from these experiments that the normal urine of cattle, sheep and guinea pigs, provided that they are healthy, is probably sterile, and that normal human urine is also probably sterile. We have, however, not yet been able to prove the sterility of normal human urine.

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